Proteomic and biochemical approaches to investigate the interactions between the Eastern Oyster, *Crassostrea virginica* and the MSX parasite, *Haplosporidium nelsoni*

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ABSTRACT

The host parasite system of the MSX parasite, *Haplosporidium nelsoni*, in the eastern oyster, *Crassostrea virginica*, poses many challenges for experimental study. The interactions that are involved in the development of the typically high prevalence and intensities of disease encountered in certain environments have not been characterized . The parasite is not-culturable and its life cycle outside of the oyster host remains unknown, as such the field is the only source of infective tissues available for study.

The main goals of this research were to first, explore of potential protein targets involved in infection; second, investigate the tissues that provide optimal comparative value and third, test methodologies that allow for clear comparative analysis. In order to investigate this host parasite system it was first necessary to develop an experimental design that enabled the consistency of samples and reliable diagnosis of their disease state. Arriving at an experimental design involved the implementation of several laboratory protocols including one and two dimensional protein gel electrophoresis, enzymatic assays, the use of two diagnostic methods, field sampling and experimental field infection, in order to determine the most promising approach to describing the host parasite interaction.

Two environmental systems in which MSX infects the eastern oyster were studied, the Bras d'Or lakes, Cape Breton, Nova Scotia, Canada and the York river system of the Chesapeake Bay, Virginia, USA. Within the Bras d'Or lakes samples, a population of oysters was discovered in which parasite pressure was present but the manifestation of disease within the tissues of oysters from this population was not found. The identification of a differential response to parasite pressure led to the comparison of proteins involved in disease from the three Bras d'Or lakes populations sampled. The new landscape of the parasite's presence in the Bras d'Or along with the targeting of several tissues and the comparison of different individuals with different disease states highlighted the need to control for variability in protein profiles.

The concentration of further comparative protein analyses to the Chesapeake Bay system allowed for the experimental field infection of naïve oysters and thus the comparison of proteins from the same individual oyster's haemolymph over time. Once collected, these samples were grouped based on final infection intensity and the comparison of protein profiles indicated the presence of a protein after exposure to an MSX impacted area. This differentially expressed protein was identified as actin and was consistently observed in the cell free haemolymph lysates from all intensity classes studied. The comparison of proteolytic activity of haemolymph over time and between final infection intensity classes identified a protease present within the initial haemolymph samples (naive individuals) occurring at a significantly higher frequency within those individuals that went on to develop high intensity infections. This finding suggests a protein differentiation found within the naïve population that impacts the outcome of infection of these individuals. The potential of this protease as a marker for heightened disease susceptibility may provide insight into the overall disease process of *H. nelsoni* within this host. Enzymatic activity also differed significantly among infection intensity classes,

as alkaline phosphatase increased over time within intensity groups, as well as comparatively between infection intensities. The monitoring of this activity may allow for the disease to be tracked more readily in populations through testing of haemolymph over time.

Having established some technical protocols that aided in clearly displaying constituent proteins from oyster tissue samples, the experimental field trial was established to compare the haemolymph of the same individual over time and exposure to MSX. This led to the identification of protein, protease and enzymatic changes associated with infection intensity. The success of this approach can aid in further characterization of infection as well as establish important indicators of the point at which disease may occur.

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Finally, the journey to complete this research was twice interrupted for the best of all reasons - the addition of my two sons to our family. Emerson and Silas, you have given me the greatest joy and the best motivation to complete this work that was started before you were born. I hope you will be able to be proud when you look at this research someday and be inspired to live the fullest life you can, knowing that with hard work you can do anything no matter how difficult it may seem.

Dedication

To Emerson and Silas

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CHAPTER 1

GENERAL INTRODUCTION

The intimacy shared between a parasite and its host species is a relationship like none other on earth. The complexity of these seemingly simple organisms in their ability to utilize the tissues, molecules and resources of a separate organism remains an area of research with many unanswered questions. In certain host parasite systems the impacts of the parasite on host population can be so detrimental as to threaten the very existence of certain species within certain geographical areas (Bremmerman and Thieme, 1989; Regoes et al., 2000; Gozlan et al., 2005; Otti and Schmid-Hempel, 2008; Rutrecht and Brown, 2008; Shirakashi et al., 2008; Rutrecht and Brown, 2009; Rigaud, 2010 et al.). One such example is the parasite commonly known as Multinucleated Spore X (MSX), *Haplosporidium nelsoni,* that affects Eastern oyster, *Crassostrea virginica*, populations of the North East Atlantic coast of North America through large scale mortalities caused by the parasite's detrimental disease processes. This parasite has eluded many attempts in characterizing the transmission, and proliferation of individual parasites following invasion of host tissues (Burreson 2004). The spread of MSX to the Canadian East coast in 2002 stimulated research trying to understand how the host-parasite interaction in Canadian waters differs from established ranges of the past half century along the shores of the Eastern US. The body of work presented here targets proteins as indicators of disease and aims to lend insight into how the parasite impacts the host at the biochemical level.

1.0 The Eastern Oyster, *Crassostrea virginica*

1.0.1 History & Distribution

Crassostrea virginica, the eastern oyster, (phylum Mollusca, class Bivalvia, order Ostreoid, family Ostreidae) is a North and South American endemic species whose range includes much of the western coast of the Atlantic Ocean (Kennedy et al., 1996). Fossil records have indicated the presence of the eastern oyster along shoals in tundra areas off the coast of North America. The eastern oyster has an established range of populations from the Maritime Provinces of Eastern Canada to the Gulf of Mexico. Reports have identified *C. virginica* as far north as the St. Lawrence River as well as its southernmost

account in Brazil, although some speculation suggests there may be distinct genetic species with similar morphological characteristics to those of *C. virginica* (Eastern Oyster Biological Review Team, 2007). Along with the natural range of the species, transplantation has occurred and certain transplant populations are also noted to exist along Western North America, the Hawaiian Islands, Japan and England (Kennedy et al., 1996).

The eastern oyster has been well documented throughout history as an important species for food and as part of the culture of First Nations' communities, as well as early settlers in North America (Lavoie, 2011; Kennedy et al., 1996; FAO, 2004; Scarfe 2006 – First Nation stakeholder). Though some accounts have reported vast populations existing within the last 150 years along the western shore of the Atlantic, historical populations can only be traced through shell deposits in many estuaries (Eastern Oyster Biological Review Team, 2007). As a food commodity, *C. virginica*, is highly regarded and sought after product within the fisheries market. While other commercial oyster species have established successful culture industries (namely, *Crassostrea gigas*, *Ostrea edulis*, and *Crassostrea ariakensis*), *C. virginica* remains a choice product that consumers and producers alike continue to rely on. In North America, the eastern oyster has seen a significant decline in both natural harvest and culture mainly due to disease, environmental and management factors (MacKenzie 2007).

1.0.2 Fishery in Atlantic Canada and the Bras d'Or Lakes

The eastern oyster has established populations within Atlantic Canada along the Northern and North Eastern New Brunswick Coastline, in Prince Edward Island and some Atlantic coastal communities of Nova Scotia. The oyster industry consists of both public beds, for which recreational leases may be purchased, and leased beds managed by the leaseholders. First Nations communities have traditional uses for the fishery, both as an income source and for food and ceremonial purposes. Harvest from both types of leases typically occurs between mid-September and the end of November when oysters have reached marketable size of a minimum 76mm shell length after an average of 2 to 4 years of growth (Lavoie 1989). Farmed oysters have not been the typical product produced

from this region, however, there is increasing farming of oysters in the region (N.Ross, personal communication). Long rakes or tongs are used for harvest, although snorkeling, diving and low tide harvest along the shallow bed habitat that the oysters prefer are also popular means used for collection.

The Eastern oyster fishery in the Bras d'Or Lakes has been recorded since 1876 (Lavoie 2011). The warmer temperatures found within the lakes system, as opposed to the exposed coastline of the North Atlantic coast of Nova Scotia, along with reproduction and seed proliferation within the sheltered low tidal pressures found in the unique system of the Bras d'Or support richer beds than those along the exposed coast. Settlement of seed on eel grass that is subsequently displaced within seasonal fluctuations causes losses of an estimated 90% of seed that is released into this environment. The remaining stocks are made up of those individuals whose settlement took place on the hard bottom of the shallow inland shores of the water system. Relay fisheries exist within the lakes allowing oysters from poor environmental areas to be placed in pristine waters in order to allow depuration and subsequent availability to the market (Lavoie 1989). The value of the Nova Scotian Eastern oyster fishery in 2001 before the MSX outbreak was valued at $1,202,811.00 (total production of 420,463 MT and 3.3% of the total aquaculture species production). Current statistics indicated a decrease of 56.2% in its value as of 2009 ($676,105 with a total production of 158,742 MT and 1.17% of the total aquaculture species production) (Nova Scotia Department of Fisheries and Aquaculture, 2011). Most of this loss can be attributed to the decline of the Bras d'Or industry following the introduction of the MSX (*H. nelsoni*) parasite.

1.0.3 Biology

Crassostrea virginica is a monomyarian bivalve, whose structure is bilaterally asymmetrical (Eble 1996). The left valve is used for settlement and will often appear notably thicker and more deeply cupped than the upper right valve. Overall shell shape and thickness are highly dependent on the substrate to which the individual oyster is attached and develops on. Hard substrates produce thick rounded shells with radial

ridges and more markedly curved umbones (thickest and oldest section of shell, found to lie dorsally in most species), while silty substrates tend to form thin slender shells with straight umbones and fewer ridges. A distinguishing character of the eastern oyster species is the purple scar where the adducter muscle attaches after metamorphosis. Overall morphology is noted as being quite variable in adults of the species due to varied environmental pressures as well as a general lack of selection pressure on adult forms, while the larval stages exhibit more constant morphological and behavioural characteristics due to pressures for long term survival, spread and success (Eble, 1996).

The eastern oyster reproductive system is rooted in the proliferation of high numbers of progeny in order to spread offspring in varied and widespread environments that are suitable for growth and establishment (Eastern Oyster Biological Review Team, 2007). Members of the species are protrandric, maturing primarily as males with a subsequent change to female in later life stages (Eble, 1996). This action is thought to be reversible in some cases. Influences on an individual's sex are quite varied and include responses to annual changes in environment, physiology and nutrition. It has also been brought forth that the sex of neighboring oysters can impact sex of individuals, with changes occurring between spawning seasons when the gonad is undifferentiated. Overall fecundity is a complex measure that is skewed by variable yet prolonged spawning periods and the gonadal tissues being incorporated into the surrounding tissues. Spawning is triggered by both temperature (above 20°C) and salinity (above 10 psu) as well as physiochemical triggers (Kennedy et al.,1996).

After fertilization, a trocophore develops and depending on environmental triggers can last in this non-feeding stage for up to two days (Thompson et al., 1996). The veliger stages are planktotrophic and can last up to two months, during which they undergo morphological changes that result in larvae with a fully developed foot aiding in migration to an appropriate settlement locale. This foot is then reabsorbed during metamorphosis into the permanently attached oyster. The larval stages utilize both passive and active transport in order to disperse in their environment; the cues initiating settlement are both environmental and internal. Settlement can be repeated but only

reversed before metamorphosis has taken place. Final metamorphosis is initiated upon response to salinity and cues emanating from biofilms and proximity to established oyster populations indicating good substrate and environmental conditions for attachment and growth. The entire process will typically be carried out over a period of 2-3 weeks after hatching, however the environmental conditions have a significant impact on progression of development. (Thompson et al., 1996)

1.0.4 Habitat and Environment

Major influences on growth and development include temperature, salinity and food availability, with temperate regions seeing maximal growth in the summer months and more southerly regions yielding faster growth and production of offspring (Shumway, 1996). The limits for salinity tolerance that have been recorded range from 0-42 psu with optimal levels ranging from 14-28 psu. The eastern oyster can survive freezing temperatures and tolerate up to 45°C but will grow and function optimally at 20-30°C. Growth in the earliest stages of life is most rapid, but slows gradually after the first six months. In standard conditions, growth can reach 15cm within 5-6 years. Harvestable oysters (measuring between 76-90mm) can be produced at different intervals depending on the conditions of the location in which they are grown (Shumway, 1996).

1.1 Pathogens and Disease of Mollusks

Many of the known pathogens and diseases of oysters, and mollusks for that matter, have been described in species that have been cultured, since stocks of cultured shellfish are heavily monitored and extensive mortalities are often investigated to identify a cause. The broader importance of these pathogens in the context of natural environments and within populations whose structure is not set up for culture (crowding, stress) is not well understood. Often the manipulations of large scale commercial fisheries confound the expression of opportunistic pathogens whose effects would otherwise be minimal in a more natural setting. Likewise, there is likely a multitude of pathogenic species that are unknown to the scientific community due to the fact that host species in which these most

readily occur are not studied with the same frequency or scrutiny that their commercially cultured cousins are. Bower (1994) compiled a comprehensive synopsis of diseases affecting shellfish with the emphasis on those which are found to occur in Canada and are of regional importance, those which are in Canada of negligible importance, as well as those that have not been reported in Canada or whose hosts are not found in Canada. The following section outlines the various threats to oyster stocks in which an immune response may be mounted and in some cases successful, and thus may be relevant to the current work.

1.1.1 Viral Diseases

Many of the popularly cultured oyster species have associated viral pathogens whose effects can be detrimental especially in a culture setting (Table 1). Currently, the Herpes virus is causing high mortalities in France (up to 50%), impacting the highly valued French oyster industry (Segarra et al., 2010, Sauvage et al., 2009, Cor Poppe, personal communication).

1.1.2 Bacterial Diseases

As seen with viral diseases, bacterial pathogens have been observed and identified mostly with cultured species (Table 2). Some may see an increase in overall impact due to the occurrence of other stressors and pathogens impacting on the same host population.

1.1.3 Diseases of Unknown Etiology

Several disease agents have eluded concrete identification despite their impacts on host populations (Table 3). One such disease noted from the Atlantic Canadian region is Malpeque disease. High level mortalities of Eastern oyster populations were first reported by Needler and Logie (1947) and later confirmed by Drinnan and Medcof (1961) in Atlantic Canada. A causative agent for these mortalities has never been established but this agent still exists within the region. Naïve oysters placed in these bays exhibit the same rapid and high prevalence mortality as demonstrated by initial reports. These oyster populations remain closed to movement outside the affected region for aquaculture so as not to spread disease to naïve stocks. Malpeque disease was found associated with

mortalities in oyster populations in the Bras D'Or lakes and Aspy Bay Cape Breton in 2007 (Vaercaemer et al., 2010; Lavoie, 2011). Attempts to identify a causative agent using PCR of random selections of tissue sections collected from control and infected animals yielded no banding differences (Lavoie, 2011).

1.1.4 Pests

Unlike pathogenic microorganisms there exists several species of aquatic macroorganisms that cause disease and mortality in oyster species through predation and utilization of oyster tissues for attachment and feeding (Table 4).

1.1.5 Commensal Organisms

The tubellarian *Urastoma cyprinae* (Burt and Drinnan, 1968) has been reported from the gills of bivalves, including *Crassostrea virginica*. While it induces pathology in the gill tissues of the mussel *Mytilus galloprovincialis* (Robledo et al., 1994), this response has not been described in oysters infected with *U. cyprinae* (McGladdery et al., 1992 and 1993). It was proposed by Burt and Drinnan (1968) that *U. cyprinae* in fact occurred as a commensal organism in *C. virginica*.

1.1.6 Parasites

Of all the pathogen groups impacting oysters, parasites appear to be the most destructive (Table 5). In many cases the effects of a parasite species is accentuated through its introduction into a naïve population often made up of a species closely related to, but not its original endemic host. Parasitic species straddle a delicate balance between taking what is needed from a host in order to survive and propagate and the need for the host to stay alive and function in order to maintain their internal environment and continue proliferation. Often a balance is achieved in many long standing host-parasite relationships over the course of generations of both host and parasite, co-evolving to the point of minimal impact (Bremmerman 1989). However, if a parasite gains access to a naïve population of hosts of a different species, the impacts of the parasite can be very grave. This is due to the host not having evolved any specified defense mechanisms toward this particular pathogen, and without an effective immune response the parasite

can often quickly overwhelm the host and cause disease and mortality within a region and/or population (Bremmerman and Thieme, 1989; Regoes et al., 2000; Gozlan et al., 2005; Otti and Schmid-Hempel, 2008; Rutrecht and Brown, 2008; Shirakashi et al., 2008; Rutrecht and Brown, 2009; Rigaud, 2010 et al.).

1.1.7 Parasitic Protozoans of Molluscs

Protozoal parasites seem the most successful of all the parasite groups found in oysters (Table 5). Their impacts vary from species to species, but it is thought that there are a great abundance of species that have not yet been discovered that occur in non-commercial oyster species worldwide.

1.1.7.1 *Perkinsus* spp.

The genus *Perkinsus* represents protistan parasites of mollusks from around the world. The life cycle of these parasites includes vegetative proliferation in hosts with trophozoite cells undergoing successive bipartitioning. There are seven valid species representatives including, *P. marinus, P. olseni, P. qugwadi, P. cheasepeaki, P. andrewsi, P. mediterraneus* (Villalba et al., 2004) and *Perkinsus beihaiensis* (Moss et al., 2008).

Perkinsus marinus or Dermo is a protozoan parasite of the eastern oyster that was first identified by Mackin 1950, from diseased oysters in Virginia, and has been studied extensively since its first report. It causes increased mortality in stocks over multiple seasons, with the second season yielding mortalities of around 50% and the third season with mortality reaching 80-90%. The gross impact on hosts includes the progression of the parasite throughout host tissues, interfering with host energy fluxes, slowing growth, and causing energy deficiencies impacting gametogenesis. The parasite's range is thought to be limited by temperature with sustained infectious populations occurring along the western Atlantic and Gulf coasts from southern Massachusetts to the Gulf of Mexico. There have been a number of reports resulting from presumed introductions in Hawaii as well as along the northern Atlantic coast including Maine. Spread of this pathogen through the Chesapeake system has resulted from the moveent of infected oyster seed (Villalba et al., 2004).

Cold temperatures can reduce overall impact of the Dermo parasite even in sustained populations (Villalba et al., 2004). It is thought that a warming trend could result in areas now minimally affected by the presence of the organism showing an increase in infection and mortalities. Interestingly, there is evidence of variations of resistance in host populations of the eastern oyster in different locales (maybe due to genetic variation among the distinct populations), as well as some evidence of parasite tolerance and pathogenicity differences to environmental conditions dependent on location (The Eastern Oyster Biological Review Team, 2007).

The lifecycle of the Dermo parasite is completed through direct transmission from infected to naïve oysters (Mackin, 1962). Spread is extensive within infected populations and mortalities resulting from infection tend to peak in summer months when temperature and salinity are at highly optimal levels, although the parasite is able to withstand wide ranges of both temperature and salinity fluctuations. The parasite thrives in high salinity waters, but even if salinity levels lower in bays where the parasite is well established, they can persist at 5psu for up to 3 months until salinity levels return to the normally high levels in these bays. Temperature is more of a concern in areas with larger seasonal fluctuations. In the Chesapeake system, both prevalence and intensity increase in late spring when temperatures rise above 20°C, these later infections often result in overwintering of the parasite (Villalba et al., 2004). The highest mortalities are seen in late August and September, while over the winter regression takes place and newly acquired infections do not reoccur until late summer the following year coincident with mortalities. Some evidence has been obtained showing that transmission can occur in rare instances without associated mortality (Ragone-Clavo et al., 2003). In areas of intense culture, Dermo disease has become managed by getting oysters to market prior to mortalities, not through mitigating the disease in the long term. Population density plays an important role in the transmission of Dermo disease due to the direct lifecycle. Age of the host seems a contributing factor as well, with older individuals experiencing more disease in the same culture areas, thought to be a result of higher filtration and longer exposure periods experienced by this group (Villalba et al., 2004).

Proposed Dermo management strategies are varied and include modified culture procedures which utilize natural environmental limitation of the disease but can be compounded by the presence of additional pathogens (e.g. MSX) in same geographic regions. Additional strategies include selective breeding and resistant strain development, encouraging increased tolerance over time due to natural selection, and triploid oyster development which provides no evidence of increased tolerance but quicker growth to market size. Genetic engineering and gene transfer is also being investigated along with allochtonous species introduction (*C. ariakensis*), and the use of chemotheraputant treatments to kill free infective cells (Villalba et al., 2004). All of the proposed strategies are accompanied by benefits and costs to natural integrity of populations, environmental risks, and overall improved production of cultured species all sources of great debate.

1.1.7.1.2 Diagnostics

The study of *Perkinsus* spp. has been greatly advanced by the ability to culture the parasite and facilitate experimental infection of oyster hosts. Ray (1966) devised a diagnostic culture method that aided in quick and accurate identification within infected populations. Subsequent propogation of the parasite from histozoic stages (Kelinschuster and Swink 1993; La Peyre et al., 1993; Gauthier and Vasta, 1995, 2002; La Peyre and Faisal, 1995; La Peyre, 1996) allowed studies of growth, stages, environmental influences, host interactions and physiology of the parasite through its lifecycle (Krantz, 1994; O'Farrell et al., 2000; Gauthier and Vasta, 2002; Brown et al., 2005; La Peyre et al., 2006, Ford and Chintala, 2006; Lund et al., 2007; La Peyre et al., 2008).

1.1.7.2 Haplosporidians

Members of the Phylum Haplosporidia are obligate parasite of a diverse number of marine and freshwater invertebrates. It comprises species of the genera *Urosporidium, Bonamia, Minchinia* and *Haplopospordium,* although species of *Haplosporidium* make up a paraphyletic grouping and revision of the genus has been recommended (Burreson and Reese, 2006; Hine et al., 2009). These parasites have a plasmodial stage and are spore forming with the ornamentation of different species' spores noted as an important taxonomic feature.

Haplosporidium armoricana occurs in France, Spain, and the Netherlands in *Ostrea edulis* and *O. angasi.* There was also a single report from Oregon in imported Olympia oysters (*O. conchaphila*) (Mix and Sprauge, 1974). Spore masses are identified as occurring in the connective tissues. Within an established culture setting the disease occurs at very low prevalence (1%) but when oysters from an unexposed population are introduced they are greatly impacted by the parasite. High prevalences and mortalities in naïve populations are likely due to the lack of defense mechanisms to fend off infection that those who have generational exposure developed over time.

Haplosporidians have been found in hatcheries of the pearl oyster *Pinctada maxima* in Australia. However those identified as harbouring infection were destroyed so impact of the parasite and strict identification of species is unknown. The parasite was noted to occur in the connective tissue of the digestive gland of those individuals identified as infected. Also in Australia, the rock oyster *Saccostrea cucullata* was found to be infected with *Haplosporidium sp.* in the report of epizootics reaching 3-27% prevalence in the northeast. Infections were often heavy with plasmodia and sporulation taking place in the connective tissue with very little defensive response noted via histology (lack of haemocyte infiltration), but an abundance of brown cells, or pigmented excretory haemocytes, were noted in these heavy infections (Hine and Thorne 2000, 2002).

1.1.7.2.1 Haplosporidium costale, SSO (seaside organism)

Haplosporidium costale is found in high salinity (above 25 psu) waters from Long Island Sound, New York to Cape Charles, Virginia. In addition, plasmodia of *H. costale* have been identified at low prevalence and intensity along the southern gulf of Saint Lawrence, the Atlantic coast, and within the Bras D'Ors lakes in Nova Scotia (DFO report not published). Within its range along the US coast, it can be associated with seasonal mortality in late spring corresponding to the sporulation of the parasite (Couch and Rosenfield, 1968; Andrews and Castagna, 1978; Andrews, 1984). The mode of transmission for *H. costale* is unknown and life cycle details including the presence or absence of intermediate host(s) is also unknown. Management of disease is sometimes

facilitated through movement of stock to areas of low salinity where the disease process is impeded.

1.1.7.2.2 Haplosporidium nelsoni, MSX

Haplosporidium nelsoni (Kingdom: Protista, Supergroup: Rhizaria, Phylum: Haplosporidia), was first reported in oysters from Delaware Bay in the fall of 1957 with massive mortalities occurring in the spring of 1958. An unknown organism identified as the causative agent of the observed mortalities was referred to as "MSX" (**M**ultinucleate **S**phere **X** unknown) (Haskin et al., 1966). Plasmodia of the parasite occur within the connective and epithelial tissues of the gills and gut and are identified through their multinucleate nature as well as their size (5-15 μm in diameter, though they can be larger) and sporulation of the species occurs within epithelium of the digestive tubules (OIE 2006). The spore surface of *H. nelsoni,* as determined by SEM, is covered with individual tightly bound ribbons occurring as a single sheet. This layer is also overlaid distally to the aboral pore by a branched fibrous network (Burreson and Reese, 2006). The mortalities were found to occur in both adult and juvenile oysters with a prevalence of up to 80% and oyster production in Delaware Bay fell from about 7.5 million pounds of oyster meat prior to 1957 to less than 100,000 pounds by 1960 (Sindermann and Rosenfield 1967).

By 1959, the disease was reported in both Virginia and Maryland waters of Chesapeake Bay (Andrews 1966) eventually leading to the identification of spore stages (Couch et al. 1966), and subsequent identification of the species *Minchinia nelsoni* (Haskin et al., 1966), which was later reclassified as *Haplosporidium nelsoni* (Perkins 1990). The mid-Atlantic coast of the United States remains the most heavily affected by MSX. The parasite's range spans along the entire Atlantic coast of the US from Maine to Florida.

Two regions outside of the established range of the parasite have been identified to sustain infections in populations, the Bras D'Or lakes, Nova Scotia, Canada (Stephenson et al., 2003) and the Gulf of Mexico. In Canada, the parasite is not found along the

Atlantic coast of Nova Scotia, but instead isolated within the unique salt water Bras d'Or lake system on the North Eastern tip of the province. This water system has a low tidal influence and is almost completely landlocked with two flushing sites at the Northern- and Southern-most reaches of the system. It may be possible that the environmental conditions in this area have allowed proliferation of the disease, either through introduction directly to the lakes, or through progression along the coast via an intermediate host occurring in or passing through areas in which the conditions were not amenable to disease establishment in the coastal populations of *C. virginica*. Isolation of *H. nelsoni* DNA from collections from the Gulf of Mexico has shown the presence of the parasite spanning the past five to ten years. However, there have been no reports of an epizootic in this area (Ulrich et al., 2007).

Molecular phylogenetics (Burreson et al., 1997) demonstrated that *Haplosporidium* parasites in endemic Asian populations of the Pacific oyster (*Crassostrea gigas*) were identical to the genetic sequences of *Haplosporidium nelsoni* isolated from Virginia. This supports the hypothesis that the origins of the initial East coast outbreak and the first reported case of *H. nelsoni* in *C. virginica* were the result of a transfer of infected *C. gigas* from the West coast that had originally come from Asian stocks. Because of the parasite's introduction into a naïve host, the eastern oyster had no evolutionary defense against the pathogen causing it to decimate stocks within environmental conditions that were ideal for proliferation. The spread of the parasite along the western coast of the Atlantic may stem from the interplay of several factors, including natural spread of the opportunistic parasite among host populations, the possibility of movement of an intermediate host throughout the range and movement of infected stocks through aquaculture.

Haplosporidium nelsoni has never been cultured, and its transmission is thought to be indirect, as it has never been determined whether or not the life cycle of the parasite utilizes an intermediate host(s). Study of naturally infected populations has been the source for all information known about the parasite and the disease process in its hosts. Infection occurs in the early summer with heavy infection being observed late in the same season in areas of repeated epizootics that have been monitored since initially outbreaks

along the mid-eastern coast of the US. The rapid proliferation of the parasite within the host's tissues occurs over approximately six weeks in many of the lethal cases. Open gross inspections show infected and diseased hosts appearing grey, watery, and emaciated. For those individuals in which the infection remains sublethal, impacts on metabolism were noted through reductions in clearance rates (Newell, 1985), condition index, fecundity (Newell 1985; Barber et al., 1988a) and glycogen content (Barber et al., 1988b) when compared with resistant individuals. Interestingly, when looking at overall energy costs in susceptible oyster populations, those with systemic infections displayed evidence of increases in clearance and oxygen consumption rates compared with uninfected individuals (Barber et al., 1991a). For those that survive the initial wave of infection, often the environmental temperatures impacted the progression of the parasite, with cooler waters slowing proliferation, as well as the metabolism of the host itself. As waters warm the following spring, a second wave of mortality is often noted consisting of those individuals whose intensity levels have remained high through the winter in the regions of study and surveillance along the Eastern US coast.

1.1.7.2.3 Environmental factors affecting host-parasite interactions

In areas where mortalities due to the parasite continue to persist, the environmental conditions seem to be an important factor impacting both the parasite's success and the host's ability to survive. Salinity is chief among these factors with drought conditions causing elevated salinity levels associated with dramatic spikes in disease within monitored areas (Barber et al., 1997). Salinity ranges above 18-22 psu are shown to consistently support infection, proliferation, and mortality, but a level of a sustained minimum of 15 psu has been shown to support infection and disease. Drought years have a greater impact due to areas of lower salinity undergoing an elevation in salinity levels, thus exposing populations normally sheltered by their environment to the conditions in which disease is favourable. Because of this, measures have been put in place to limit transfer of oysters from impacted areas to those of lower salinities. In those areas in which drought conditions have resulted in spread, it has been found that with the absence of drought conditions these populations are able to recover and their progeny survive and produce healthy populations (Barber et al., 1997). This was demonstrated through the

1970's in both Delaware and Chesapeake Bays during which time wet environmental conditions corresponded to a significant recovery of the eastern oyster population that had fallen victim to the initial parasite outbreaks in the 1950-1960s. Many of the oyster recovery programs have focused on areas with natural salinities of low to moderate levels.

1.1.7.2.4 Comparison of MSX and SSO and Diagnostics

Plasmodia of *Haplosporidian costale* cannot be distinguished through histological analysis from those of the parasite *Haplosporidium nelsoni.* However, the site of sporulation differs with *H. nelsoni* undergoing sporulation in the epithelium of the digestive tubules, while *H. costale* undergoes synchronous sporulation throughout the connective tissues. The seasonality of the parasites also differs, with the mortalities occurring earlier in the season for *H. costale*. However, plasmodia of *H. costale* were detected in October in Long Island Sound and Virginia, which challenges the traditional knowledge of the parasite's seasonality (Stokes and Burreson 2001; Sunila et al., 2002). The co-detection of *H. costale* and *H. nelsoni* is facilitated through PCR diagnostics as well as *in-situ* hybridization, which overcomes traditional histological limitations.

1.2 Host Response

The innate immune systems of molluscan hosts implement two main streams of defense. Cellular defenses include phagocytosis, apoptosis, respiratory burst and diapedesis, while humoral defenses utilize secreted molecules such as protease inhibitors, lysozymes, lectins, aminopeptidases, and antibacterial proteins (Goedken et al., 2005).

1.2.1 Innate Immune Molecules

Lysozyme is an enzyme utilized in innate immune system responses, acting by catalyzing the hydrolysis of 1,4-beta-linkages shared between N-acetylmuramic and N-acetyl-D-glucosamine residues found in peptidoglycan as well as a similar bond in fungal chitin (Jolles, 1996). As a result, lysozyme is an effective defense against many bacterial species, particularly Gram positive groups. Lysozyme has been identified from in many species and is found to occur in secretions such as tears, saliva, plasma, and mucous

(Jolles, 1996). In marine species, lysozyme has been measured mainly in serum, plasma, or heamolymph as a measure of circulating enzyme indicative of a proinflammatory phagocyte response (Lie et al., 1989; Saurahb and Sahoo, 2008, Fange et al.,1976). In mollusks, lysozyme has been characterized from a number of species (Xue et al., 2010) however, its specific action within these species has not been fully characterized, nor has its optimal conditions for action (particularly the pH range of activity within these systems).

Alkaline phosphatase is an enzyme whose action serves to remove phosphate groups from proteins and other molecules including nucleotides (Crofton, 1982). It is most effective in basic environments and has been described from many groups of organisms. In bacteria the enzyme is thought to act in acquisition of phosphate groups from molecules when phosphate is in low abundance (Sebastien and Ammerman, 2009). It has been proposed that alkaline phosphatase can also act in aiding in the uptake of organic molecules by removing phosphate groups that tend to impede uptake by bacteria. The role of alkaline phophatase in immune function is not clearly understood, though in a number of organisms and disease interactions it has been noted to be upregulated. An increase in alkaline phophatase is thought to indicate stress or a trigger for an immune response (Ross et al., 2000; Iger and Abraham, 1990, 1997). In mollusks, the release of alkaline phophotase in addtion to othe rlytic enzymes has been demonstrated after the formation of a phagolysosome in the reponse to microorganism infection (Gestal et al. 2008; Sokolova, 2009).

Proteases occur naturally in all organisms and act to break down proteins through hydrolysis of peptide bonds between amino acids in a polypeptide chain. There are numerous proteases that have been described and classified into four broad groups (serine, cysteine, aspartate, and metallo- proteases) (Hartley 1960). Proteases serve many physiological functions from digestion of protein as food to more regulated cascades affecting complement, apoptosis, blood clotting and the invertebrate prophenoloxidase activation (Morrissey, 2008; Cho et al., 2002). Proteases can act in breaking specific bonds or breaking an entire protein down into its constituent amino acids. Similarly,

proteases can detach terminal peptide bonds disrupt the polypeptide chain at an internal linkage. The action of proteases can serve to trigger rapid changes in metabolic and immune functions in response to physiological perturbations within an organism.

Additional molecules which have been shown to have a role in host response and immune function include flavoenzymes which act as catalysts in a variety of chemical reactions (Joosten and vanBerkel, 2007), and transferrin which binds iron molecules and has a role in the innate immune system in mucosa impacting iron availability and thus bacterial survival (Stafford and Belosevic, 2003). Actin has been found at significant levels in Atlantic salmon mucus, which raises a question whether it may have an alternate extracellular role in organisms similar to histone (Easy and Ross, 2009). Actin is a 42kDa protein that is ubiquitous in all eukaryotic cells and acts as a component of micro- and thin filaments utilized in the structure and motility of cells. Actin plays an important role in many cellular functions including cell motility, shape, division, cytokinesis, muscle contraction, signaling, and the creation of cell junctions (Pratt et al., 2004a,b). Lectins (hemagglutinin) are highly specific sugar binding molecules that serve a variety of physiological functions in a wealth of organisms. Their specificity is implicated in immune function, recognizing carbohydrates found on the surface of pathogens. In mollusks, their immunological roles include non-self recognition, induction of phagocytosis and encapsulation anti-bacterial defense and microbe agglutination (Wang et al., 2011). C-reactive proteins (CRPs) are found in the blood an act in binding phosphocholine on the surface of dying cells and trigger activation of complement. Inflammation is associated with a distinct rise in CRPs. Apolipoprotein A-I is a lipid-binding protein that assists in the transport of hydrophobic lipids through lymphatic and circulatory systems. Antimicrobial proteins and peptides are a diverse group of peptides that constitute a highly conserved defense mechanism in innate immune systems. They can effectively kill bacteria, fungi, enveloped viruses and have been demonstrated to kill transformed cancer cells. Their action is achieved through disruption of membranes, cytoplasmic components or metabolic processes. They can also act as important immunomodulatory molecules with the ability to alter host gene expression, impact

production of chemokines, modulate adaptive immune cells, encourage wound healing and inhibit cytokine production (Hamill et al., 2008).

1.2.2 Evidence of Oyster Host Response to Non-Parasitic Pathogens

Oyster host response has been studied in several disease systems, lending insight into how this bivalve deals with infiltrating pathogens. Freidman et al. (1999) noted that when challenged with *Nocardia* sp., heat shock proteins were detected in oyster hosts. Although the pattern of synthesis (protein production) was found to be similar to control animals, overall thermotolerance was reduced in those oysters exposed to nocardiosis. Species of *Vibrio* differ in pathogenicity, but typically young oysters are unable to mount a defense against infection, while as oyster hosts age the act of pathogen sequestration seems a successful means of controlling infection. Nottage et al. (1989) identified two low molecular weight toxins playing a role in disease progression, a proteinase (40,000 Da) involved in degradation of connective tissue, as well as a ciliostatic toxin (500-1000 Da). *Vibrio aestuariamus* produces ECPs (extracellular products) with hemocyte immunosuppressant activities. Experimental inoculation of oysters (*Ostrea edulis*) with heat killed *M. luteus, V. splendidus,* and *V. anguillarum* displayed an elevation in cDNA transcripts of an Interleukin-17 homologue (CgIL-17) in hemocytes, suggestive of an early response at the gene expression level toward these pathogens.

Host species are often quite adept at counteracting effects of shell boring sponges through rapid nacre (inner shell surface) application to repair shell damage. However, if this repair response is slowed due to other stressors, the sponges can overcome the host and cause mortality (Moase et al., 1999). This problem is of specific concern to the pearl oyster industry in that nacre application is diverted to shell repair, thus slowing nacre application onto embedded pearl (Moase et al., 1999). Management can mitigate impact of this pest through the use of hanging as opposed to bottom culture of species.

1.2.3 Host Parasite Interactions

Responses of oysters to parasites are of particular interest due to their impact on molluscan hosts compared to other pathogens. Metacestode infections can result in high prevalence with every individual in some populations harbouring many (up to several

hundred) parasites (Lauckner, 1983, Winstead et al., 2004). In the presence of such heavy parasite burden, the host can suffer from generalized physiological stress-impacted growth and reproduction (Sparks, 1985), or may show no distress whatsoever from infection (Lauckner et al., 1983).

The lifecycle of *Marteilia* spp. is likely to involve an intermediate host with experimental studies not providing evidence of direct horizontal transmission. Audemard et al. (2002) used molecular techniques to show *M. refringens* in ovarian tissues of the copepod, *Paracartia grani,* indicating the potential involvement of this species in the lifecycle. However, attempts at producing transmission in the lab were unsuccessful using this model. Infections occurring in *C. gigas* have been shown to be transient and produce little impact on culture populations of this species. The results of infection in other species are initiated by primary cells in the epithelium of the gut or gills, subsequent sporont development and sporulation take place in digestive gland epithelium (Bower, 2006).

With *Marteilia sydneyi,* similar to what has been found in MSX, seasonality includes a point at which low temperatures cause overwintering of parasites within the host's tissues and when temperatures rise in the spring, mortalities of these infected individuals follow. However, there has not been correlation established between epizootics and fluctuations in pH, salinity, and temperature. *Marteiliodes chungmuensis* infections have been found to reduce overall serum protein concentrations affecting metabolic recovery after spawning (Park et al., 2003; Park, 2005).

Bonamia perspora sporulation causes disruption of the digestive diverticula and interestingly, haemocyte infiltration is found to be strongest within those infections with uninucleate and binucleate microcells and weakest in individuals that have developed plasmodial or sporulating forms through the course of infection (Carnegie et al., 2006).

Urastoma spp. displayed a preference for oysters compared to other Atlantic Canadian molluscan species (Brun et al., 1999), indicating an attraction to mucous secretions in oyster gills, most heavily concentrated along the basal food tract. Despite no specific pathology among oyster hosts being noted as numbers of *U. cyprinae* increase, there is

likely to be impact on the overall condition of the oyster. Indeed, *U. cyprinae* has been demonstrated to affect oyster mucus proteolytic activity (Brun et al., 2000).

1.2.3.1 Host Parasite Interactions – Cellular Immunity

The host parasite dynamics of protozoal infections most common in *Crassostrea virginica* are of great interest, especially in further understanding the disease processes caused by these pathogens. Histological examination of MSX infected oysters has noted that infiltration through circulatory systems and subsequent proliferation in the digestive and connective tissues has a detrimental effect on cellular functions of respiration and feeding (Barber et al. 1988b). Little has been elucidated regarding the cellular actions taking place in these tissues during infection however. Fecundity has also been notably repressed by infection, but may simply be a byproduct of energy reallocation of host reserves to defense mechanisms (Barber et al., 1988). Barber et al. (1988) also showed a decrease in lipid, glycogen, protein and ash content of those oysters carrying systemic MSX infections.

With respect to *Perkinsus marinus*, whose study has been advanced both *in vitro* and *in vivo* due to culture techniques being available, a more detailed view of cellular interaction has been established. *Perkinsus marinus* has been shown to effectively utilize the cells that have phagocytised the pathogen in order to circulate through the host and establish systemic infections. It is also thought that they are able to produce superoxide dismutase and through this action effectively counter the effects of the ROIs (reactive oxygen intermediate free radicals) released to kill pathogens during phagocytosis (Ahmed et al., 2003). The parasite targets the immune cells of the oyster inhibiting the host response due to the destruction of haemocytes impeding defense systems against this and other opportunistic invaders. The parasite is phagocytized by host haemocytes, in which they proliferate and spread by infiltrating various tissues through movement of the host's cells (Caseres-Martinez et al., 2008). Initial infiltration typically commences within the epithelium of the gut (Mackin, 1951), gill, labial palps, or mantle (Chintala et al., 2002).

Increased haemocyte activity has been shown to be a determining factor in molluscan resistance (Fisher and Newell 1986), with activities typically decreasing in infections with *P. marinus* (LaPeyre and Faisal,1995). However, this indicator has proved somewhat problematic when assessing impact of the pathogen, due to haemocyte numbers varying among species and individuals dependent on age, heart rate, bleeding technique, environmental temperature and air exposure (Adema et al. 1991, Thompson et al., 1978). Because of this, it is understandable that increased activity in oysters is noted with those haemocytes being infected often lysed through the course of disease, therefore the observation of increased haemocyte activity may be an artifact of reduced numbers of haemocytes found in infected individuals. In contrast, a decrease in circulating haemocytes was noted for clams whose cells are diverted toward encapsulation to contain the disease (Casas 2002). The host species seems an important factor, with *C. gigas* displaying some resistance to *P. marinus.* Comparative studies of the two oyster host species have not determined clear differences, except that rate of phagocytosis with *C. virginica* is greater during infection than for *C. gigas* (Gauthier and Vasta, 2002), which may be due to temporal activation of oyster defense systems caused by previous exposure to the pathogen (Chu, 1988; Ford, 1988; Gaffney and Bushek 1996). This may indicate that *C. gigas* is able to utilize defenses other than phagocytosis and thus block proliferation of the parasite, or simply has an internal environment that does not favour disease.

Immune responses vary with hosts, typically involving phagocytosis or encapsulation by haemocytes and environmental factors are also known to impact disease progression and regression by modulating host immune systems and parasite activity (Chu and LaPeyre, 1993; Anderson, 1996). Those *Perkinsus* sp. found in clam and abalone are successfully controlled through encapsulation, during which parasites apparently die while embedded within the macromolecule formed through non-glycosylated polypeptides secreted by surrounding haemocytes (Chagot et al., 1987; Montes et al., 1995; Sagrista et al., 1995; McLaughlin and Faisal, 1998).

1.2.3.2 Host-Parasite Interaction – Humoral Immunity

Many studies of *H. nelsoni* have focused on humoral factors in oysters and have reported declines in free amino acids (Feng and Canzonier, 1970) as well as total serum protein concentrations associated with systemic infections (Ford, 1986). When hemocytes were incubated along with *H. nelsoni* plasmodia pre-treated with several different classes of inhibitory proteins, Ford (1988) found increased phagocytosis to be associated with those plasmodia that had been treated with carbohydrases, proteases and glycolysis inhibitors. This provided evidence of phagocytosis interference by the parasite through surface modifications and likely more importantly metabolic production of molecules inhibiting phagocytosis by host cells.

The roles of specific humoral factors in the host-parasite system of Perkinsus are still not clearly understood. Host lysozyme and haemolymph agglutination are reduced by the presence of certain extracellular products and proteases secreted by the parasite. In some systems, host lysozyme and haemolymph agglutination are reduced by the presence of certain of the parasite's extracellular products and proteases, while in other systems certain host antiproteolytic factors indicate specified action against the parasite's proteases and suggest an important role in defense (Faisal et al., 1998, Oliver et al., 2000). However, when investigating several candidates for host defense, lysozyme, lectins, and overall serum protein concentrations showed no link to resistance or pathology of disease (Chu and LaPeyre, 1989; Chintala et al., 1994). Polypeptides isolated from clam haemocyte (*Tapes philippinarum*) secretions have shown effectiveness in killing *P. marinus*. While *in vitro* studies have shown that *P. marinus* secretes proteases involved in host tissue degradation, the parasite also has the ability to suppress toxic oxygen radicals produced by host haemocytes. Extracellular products of perkinsus inhibit haemocyte activity *in vitro*; additional secretory products also inhibit production of superoxide anions by oyster haemocytes, thus modulating respiratory burst activity (Garreis et al., 1996, Anderson, 1999).

The impact of *Perkinsus* spp. on haemolymph lysozyme concentrations are uncertain, with some reports showing no difference among infected and uninfected individuals, but

seasonal variations with temperature and salinity have been reported (Chu and LaPeyre 1989, 1993b; Chu et al., 1993). Decreases in lysozyme activity were reported from infected oysters (LaPeyre et al., 1995) and serum treated with *P. marinus* ECPs (Garreis et al., 1996), while Chu and Le Peyre (1993a) reported serum lysozyme activities increased in infected oysters. These changes may be attributable to environmental factors, although mussel lysozyme had an inhibitory effect on *P. marinus* growth *in vitro*, far greater than serum lysozyme isolated from *C. virginica* (Anderson and Beaven 2001). However, contributions of other humoral factors cannot be ruled out.

Overall serum protein concentrations have been reported to be slightly lower in *P. marinus* infected oysters though not shown to be significantly different (Chu and LaPeyre, 1993; LaPeyre et al.,1995), and some reports have indicted no differences seen in oysters (Chu and LaPeyre 1989, 1993). Again, as with lysozyme, there could be a number of factors contributing to the makeup of overall protein serum concentrations. The increase noted in clams infected with *Perkinsus atlanticus* could be due to specific polypeptides reported by Montes et al. (1996, 1997).

Agglutinins may increase phagocytosis acting as opsonins (Olafsen et al., 1992), with the agglutination of non-self particles acting as a precursor to recognition and followed by internalization of the material by haemocytes (Chu, 1988). ECPs have an inhibitory effect on oyster haemagglutination titers (Garreis et al., 1996), with serum agglutinin levels not dependent *in vivo* or *in vitro* on infection intensities. Chintala (2002) concluded no role of agglutinins in oyster (*C. virginica*) defense against *P. marinus,* while in clams (*Ruditapes decassatus*) and *C. gigas* higher levels of agglutinins have been demonstrated in infected (with *P. olseni* and *P. marinus,* respectively) versus uninfected individuals (Ordas et al., 2000; La Peyre et al., 1995).

Parasite derived proteolytic enzymes serve many roles in infection processes such as adhering to host cells (Aliva and Calderon, 1993), penetration and digestion of host tissues (Sung and Dresden, 1986; McKerrow, 1987; Knox and Jones, 1990; White et al., 1996; Berasin et al., 1997; Perkins et al., 1997) and evasion of immune response (Ellis

1981; Kamata et al., 1995; Garreis et al., 1996). The release of lytic enzymes to modulate response and increase infection intensity has been suggested, but these specific enzymes remain uncharacterized (Garreis et al., 1996; LaPeyre, 1996; Anderson, 1996). ECPs and proteases reduce motility of haemocyte cells likely triggered by recognition and uptake of the parasite. Gauthier and Vasta (2002) demonstrated that oyster haemocytes displayed higher affinities toward live rather than fixed cells. Parasite-derived proteolytic enzymes may contribute to necrosis, causing degradation of the extracellular matrix components of stroma and basal membranes, further facilitating invasion of host tissues (Villalba et al., 2004). Homogenate extracts of plasma from *C. virginica* exposed to *P. marinus* cultures increased protease secretion, which was not seen to occur in extracts from less susceptible species (MacIntyre et al., 2003).

Protease characterization from ECPs of cultured *P. marinus* taken from *C. virginica* and *M. arenaria* showed primarily serine proteases that were found to be stable at high pH (LaPeyre et al., 1995, Faisal et al., 1999). Additionally, high α-chymotrypsin and low trypsin activities were detected in *P. marinus* cultures. Serine proteases are involved in life cycle development of protozoa and blood cell invasion in fish, their effects can be lethal (*Aeromonas hydraphila*), which would lend credence to why some *P. marinus* secretion products (serine class) affect several immune parameters of oysters (Garreis et al., 1996) and degrade certain host serum proteins (Oliver et al., 1999). *P. marinus* serine proteases suppress vibriocidal actions of oyster haemocytes, making hosts more susceptible to secondary infections (Tall et al., 1999). Lack of (or undetected production of) serine proteases by *P. olseni* and *P. atlanticus* could support the theory of lower virulence of these found in *T. decustatus* than that of *P. marinus* in its host *C. virginica* (Casas, 2002).

Antiproteases are found in animal serum including mollusk species, and can act in defending host cells against protozoal entry. The serine protease inhibitor family (serpin superfamily) is involved in insect defense against pathogen proteases. *P. marinus* infected oysters have antiproteases with specific activity against parasite ECPs (Faisal et al., 1998; Oliveret al., 1999a,b). Disease intensity was found to negatively correlate with

protease inhibitory activity (Oliver et al., 2000), with highest concentrations observed just before parasite elimination. This suggests a role for oyster antiproteases in protecting hemaglutinins from degradation by parasite proteases (Romestead et al., 2002). *C. gigas* which is less susceptible to the parasite displayed much higher inhibitory protease activity then that of *C. virginica* (Faisal et al., 1999), while in clams, production of specific proteins by haemocytes was found in a mounted defense response against the invading parasite, *Perkinsus atlanticus* (Montes et al., 1995, 1996, 1997).

Parasite-derived acid phosphatase plays a role in host immune response (Volety and Chu, 1997) as an enzyme that may alter bivalve cellular defense activity by disruption of phosphoproteins and inhibition of superoxide anion production. Living *P. marinus* show a negative effect on haemocyte respiratory burst activity (measured by chemiluminescence) and can actively suppress active oxygen radicals, thus interfering with host oxygen dependent killing mechanisms. Two iron-containing superoxide dismutases (Fe-SOD) and antioxidative enzymes have been described and characterized from cultured *P. marinus* (Wright et al., 2002, Ahmed et al., 2003, Schott et al., 2003). *In vitro* experimentation showed *P. marinus* caused decreases of phenoloxidase activity in haemolymph of *C. virginica* and non-susceptible mussel species *G. demissa* (Deaton and Jordan, 2002). The role of phenoloxidase in the insect arthropod immune response is well documented; however its role is currently unknown within molluscan haemocyte defenses. Continued investigation of lipid metabolism of *Perkinsus* spp. (Soudant and Chu, 2001, Lund and Chu, 2002, Chu et al., 2003) and should provide additional insight into host parasite interactions of this relationship.

1.3 Aims of Present Research

The research undertaken in this thesis set out to build on the foundational work that has followed from the initial characterization of *Haplosporidium nelsoni* in the Eastern United States. With the appearance of *H. nelsoni* in Canadian waters, there was an opportunity to investigate this parasite within a new geographically distinct environment and look for factors that may lend insight into how the parasite spreads and behaves within this setting. Through establishment of the present work in assessing the population

at risk in the Bras d'Or lakes, differences in disease expression across localities in the lakes were identified. Despite the well established limitations of studying this parasite without the ability to culture or even isolate the parasite from its host for study, an approach targeting a comparison of overall protein profiles of infected and uninfected hosts was determined as a starting point for investigation. Investigating the whole proteome of infected individuals in comparison with uninfected oysters would highlight major changes in protein expression for investigation in relation to disease status. Proteins of interest would warrant further investigation as playing a potential role in the host-parasite interaction. Further investigation led to experimental field infections of oysters in Virginia waters to look at protein profiles of individual oyster responses to disease exposure over time. The experimental design involved collection of naïve oysters and, after the collecion of a small heamolymph sample from each individual, these oysters were transplanted to a disease impacted area of the York River system of Virginia. Subsequently, additional heamolymph samples were collected over time and their profiles compare with the initial samples taken from each invidual. At the final sample time, infection intensity was determined for each individual and oysters were grouped based on these intensities for proteomic comparison. The work presented offers new insight into the actions of disease within this host parasite system and how that may differ from simply becoming infected. Specific target proteins have been identified and correlated to disease progression over time. Finally, this study offers a new avenue of research to continue with in order help elucidate more clearly the actions of this biologically elusive parasite.

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Table 1. Summary of viral diseases impacting oyster species.

Virus	Hosts	Distribution	Impact	Reference
Oyster velar virus	*Crassostrea. gigas*	Washington State	An icosahedral DNA virus found in the velar epithelium of larval stages, impacting hatchery yield	Elston 1979.
Hemocytic infection virus	*C. angulata* and *C. gigas*	France and Spain	Icosahedral DNA virus	Renault and Novoa 2004
Gill disease	*C. angulata* and *C. gigas* some signs of clinical expression in co-habitating *Ostrea. edulis*	France, Portugal, Spain and Great Britain	Produces yellow spotting on the gills and is associated with gill erosion leading to high levels of mortality.	Alderman and Gras 1969; Farley 1978
Papova-like virus	*Pinctada maxima*	Australia	Found as virus like particles in hosts, displays some similarity to viruses found in *C. gigas* and the clam species *Mya arenaria*	Norton, Shepherd and Prior 1993
Akoya virus	*Pinctada fucata, P. martensii, P. margaritifera, C. gigas*, and the scallop species *Chlamys nobilis*	Japan, China and French Polynesia	Subject of some debate relating to the proper identification and overall pathogenicity.	Comps et al. 1999 ; Miyazaki et al., 1999 ; Renault and Novoa 2004
Herpes type virus	*C. virginica, C. gigas, O. edulis, O. angasi, O. chilensis*	Maine, NY, New Zealand, France, California, Mexico and Australia	Thought to have resulted from interspecies transmission, typically found to occur in conjunction with other stressors including the presence of other disease agents, poor husbandry and crowding in culture.	Comps and Cochennec 1993; Arzul et al 2001 a and b; Davison et al. 2005.
Viral gametocytic hypertrophy, caused by a papilloma type virus	*C. virginica*, similar unconfirmed reports are noted from several other species.	Atlantic Canada, the Eastern US, North Eastern Florida coast, with possible reports on the West coast of the US, Korea and Japan	Disrupts gametes and epithelial gametogenic tissue caused by replication of the virus in host cell nuclei. Typically found at low intensities but high prevalence. overall impact on fecundity and health is not of great concern, light haemocytic infiltration is documented as a mounted host response	McGladdery et al. 1993; McGladdery et al 1999; Choi et al 2004; Farley 1985; Garcia et al 2005; Winstead et al. 2004

Table 2. Summary of bacterial diseases impacting oyster species.

Bacteria	Hosts	Distribution	Impact	Reference
Nocardiosis, Actinomycete bacteria, *Nocardia crassostreae*	*Crassostrea gigas* and *Ostrea edulis*	North American West Coast as well as Japan	Causes infection year round with associated mortalities noted in the late summer months. Characterized by yellow and green pustules on tissues of the mantle, gill, adductor muscle and heart.	Freidman et al 1998
Vibriosis, by numerous species of the genus Vibrio	*C. virginica, O edulis, C. gigas, C. sikamea, O. conchaphila* and other cultured juvenile mollusks.	Eastern US as well as California, Washington State and Japan	Impacts bivalve hatchery and nursery practices through colonization of the peripheral valve margin and spreads through mantle and soft tissues of larval and juvenile stages. Causes widespread necrosis, release of toxins and in many cases death. Presumptive diagnosis achieved through isolation of gram-negative rods from sample.	Elston et al 2008
Rickettsia- and Chlamydia-like	All oyster species as well as a plethora of other marine mollusks		Occurs as microcolonies in the gill and digestive gland epithelia, ubiquitous with light intensity infections and no associated pathology, except for one report from the French coast in the 1990s of Chlamydia being detrimental to stocks.	Renault and Cochennec 1995; Comps 1983
Juvenile oyster disease, α-Proteobacteria, Roseovarius crassostreae	*C. virginica*	North Atlantic coast of the US	Syndrome causing morbidity and mortality, peaking in high temperatures of July and August.	Barber et al 1996 ; Boardman et al 2008 ; Maloy et al 2007
***Cytophaga* spp.**	all cultured species	ubiquitous	Causes hinge ligament disease, characterized by the break down of the ligament impacting both respiration and feeding. Secondary infections often occur when disease has set in, elevated temperatures display enhanced pathogenicity.	Dungan and Elston 1988; Dungan et al 1989

Table 3. Summary of diseases of unknown cause impacting oyster species.

Disease	Host	Distribution	Impact	Reference
Digestive tract impaction	Larval *Crassostrea gigas*	Washington and Australia	Dermocystidium-like relation of protists.	Handlinger 1999
Hemocytic neoplasia	*C. virginica, C. gigas, C. iredalei, Ostrea edulis, O. conchaphila, Saccostrea commercialis, Tiostrea chilensis,* does not occur in oysters in Canadian waters but is found in Canadian mussel and clam hosts	Ubiquitous	Associated with a low prevalence, with pockets of higher prevalence in a few distinct populations. Typified by the appearance of neoplastic hemocytes in the soft tissues, thus impacting normal hemocyte function	Balouet et al 1986; Harshbarger et al 1977; Barber 2004
Malpeque Disease	*C. virginica*	Atlantic Canada	High level mortalities of Eastern oyster populations in Atlantic Canada. Pockets of disease still exist within populations of resistant oysters.	Needler and Logie 1947; Drinnan and Medcof 1961

Table 4. Summary of pests and commensal organisms impacting oyster species.

Pest/Commensal	Host	Distribution	Impact	Reference
Bivalve inhabiting hydroids, cnidarians comprising numerous species of the genera Eugymnanthea and Eutima	*Crassostrea gigas, C. rhizophorae, C. virginica*, and some species of Ostrea.	Japan, the Mediterranean Sea, Puerto Rico and Florida	Attachment occurs on the soft tissues of the mantle cavity.	Kubota 2000; Winstead et al 2004
Shell boring polycheates, spionid species of Polydora as well as species of Boccardia	*C. virginica, C. gigas, O. edulis, Saccostrea glomerata* and other bivalve species including mussels, scallops and abalone	Global distribution, with individual species displaying range limitations	Tend to be innocuous with the animal burrowing only into the shell surface, however, in Eastern and Southern North America *P. websteri* and *P. ligni* in *C. virginica* cause burrows that continue down through the shell resulting in blisters and abscesses in the host adductor muscle, greatly impacting marketability of these individuals	Bower, 2004
Shell boring sponges, *Cliona*	*C. virginica, C. gigas, O edulis, Pinctada maxima* and a variety of other oyster and bivalve species (including scallops and mussels)	Worldwide distribution, with specific species of limited range	Burrow through the periostracum and can form a network of tunnels. Can result in penetration through to the conchiolin layer to gain access to the inner host surface	Bower, 2004
***Boonea* spp. and *Odostomia* spp from the gastropod family Pyramidellidae**	*C. virginica* and *O. edulis* as well as many mussel, clam, cockle and scallop species.	Ubiquitous with most species occurring within the waters of the North Atlantic Ocean.	Snails attach close to the mantle edge, and then use their stylet and proboscis to penetrate the soft tissue and feed off oyster tissue fluids. Found to transmit *Perkinsus marinus* in *Crassostrea virginica*.	Bower 2004; White et al 1987
Tubellarian *Urastoma cyprinae* (commensal organism)	*Crassostrea virginica*	Atlantic Canada	Reported from the gills of bivalves, inducing pathology in the gill tissues of the mussel *Mytilus galloprovincialis*, this response has not been described in oysters. Proposed as a commensal organism in *C. virginica.* Evidence of altered mucoid proteolytic activity (Brun 2000).	Robledo 1994; McGladdery 1992,1993; Bower 1994; Burt and Drinnan 1968; Brun et al 2000

Table 5. Summary of parasitic diseases impacting oyster species.

Parasite	Host	Distribution	Impact	Reference
Echinocephalus crassostreai	*Crassostrea gigas, C. virginica*, abalone and some sea urchins	Hong Kong, China and Louisiana	Occurs when second and third larval stages of *Echinocephalus crassostreai* encyst in the gonad, resulting in limited associated pathology in the bivalve host, but if ingested by humans, can cause gastric or other forms of granulomatous cysts.	Cheng 1978; Bower 2004
Unidentified metacestode parasite species of *Tylocephalum*	*C. virginica ,C. gigas, C. madrasensis, Saccostrea glomerata, Striostrea mytiloides, Pinctada sp.,* scallops, clams	Tropical and subtropical waters in the Gulf of Mexico, Hawaii, Japan, Taiwan and India	Oysters and other plelecypods serve as the primary intermediate host, Molluscivourous gastropods and some fish and crustaceous species serve as the secondary intermediate or paratenic hosts, while the definitive hosts of all representative species are thought to be elasmobranches.	Laukner 1983; Bower 2004
***Mytilicola intestinalis*, red worm disease, parasitic copepod**	*Ostrea edulis* and *C. gigas*	European waters	Will not readily infect oyster species in the presence of mussels.	Cheng 1967
Oyster egg disease, undescribed protozoan (possibly microspora)	*C. gigas, C. echinata, O. edulis*, and *Saccostrea commercialis*	California, Japan, Korea, Australia, and France	Typically found at low intensities, may utilize vertical transmission occurring within the cytoplasm of mature oysters' ova, induces both haemocyte infiltration and necrosis.	Bower 2004
Apicomplexan parasites	*O. chilensis*	New Zealand	Zoites associated with infection with the parasite *Bonamia exitiosia*, possibly increasing sensitivity through occupation and destruction of haemocytes, disrupting connective tissues and utilizing host glycogen reserves.	Hine 2002
Unidentified species of Bonamia parasites	*O. angasi, O. chilensis, O. puelchana*, and *C. ariakensis*		Experimental trials conducted in *Bonamia* sp. from *C. ariakensis* indicate the role that low salinity (below 30psu) may play in limiting the disease	Audemard 2005, 2008 a, b
Unidentified protistan parasite	*P. maxima*	Western Australia	Sporozoans, thraustochytridea, ciliates in digestive gland epithelia, as well as intracellular ciliates in the epithelium of the digestive gland	Bower 2004
Unidentified protistan parasite	*P. maxima*	Western Australia	Sporozoans, thraustochytridea, ciliates in digestive gland epithelia, as well as intracellular ciliates in the epithelium of the digestive gland	Bower 2004

Parasite	Host	Distribution	Impact	Reference
Microsporidosis	Dredge oyster	New Zealand	Oval cysts in the connective tissues surrounding the gut and which contain numerous spores	Jones 1981
Gregarine parasites, *Nematopsis ostrearum, N. prytherchi,* and *N. legeri*	Not only oysters but many marine bivalves including mussels, clams, cockles and scallops	Occur ubiquitously with each species having defined distributions	Gymnospores and oocytes or naked sporozoites occur in phagocytes and subsequently move into connective tissues of the organs. The initial observation in the gills and an associated focal inflammatory response often noted as being benign. Completion of the lifecycle does not include multiplication in the bivalve host but instead within final arthropod (crab) hosts.	Bower 2004
Gill trichodinids (*Trichdina* spp.)	*C. gigas, C. angulata*, along with clams, cockles and scallops	Europe the Eastern US and Pacific rim	Occur at low intensities attaching to the mantle, labial palps, and gill.	Bower 1994
Marteiliosis (aber disease) also referred to as digestive gland disease, caused by Marteilia refringens (Phylum Paramyxea).	*O. edulis, Mytilus edulis, Cardium edule, Crassostrea virginica, C. gigas, O. chilensis, O. angasi, O. puelchana*	Atlantic Europe including southern Britain to Portugal, Morocco, Greece, Florida, Persian Gulf, and Australia	Associated with serious impacts on the European culture industry of *O. edulis* since 1968. Results of infection unpredictable, some oysters can be infected without causing disease.	Berthe et al 2004
***Marteilia sydneyi*, QX disease**	*Saccostrea glomerata, Striostrea mytiloides, Saccostrea forskali* and similar impacts in giant clams	Australia	Exposure time is thought to be relatively short about 2 weeks, after which warm temperatures favour parasite proliferation.	Lester 1986; Anderson et al. 1994; Wesche 1995; Adlard & Ernst 1996
Marteilia-like parasite	*Ostrea angasi*	Australia	Similar to the paramyxean protists of France and Australia, has been found on two occasions at very low prevalences	Heasman et al 2004
***Marteiliodes chungmuensis* (phylum Paramyxea)**	*C. gigas, C. nippona* (after transplantation), *C. echinata*, and manila clams	Korea, Japan and Australia	Infected individuals exhibit abnormal egg masses with nodular appearance.	Itoh 2002
***Marteiliodes chungmuensis* (phylum Paramyxea)**	*C. gigas, C. nippona* (after transplantation), *C. echinata*, and manila clams	Korea, Japan and Australia	Infected individuals exhibit abnormal egg masses with nodular appearance.	Itoh 2002

Parasite	Host	Distribution	Impact	Reference
Marteiliodes branchialis	*Saccostrea commercialis*	Australia	Presenting as focal lesions on gill lamellae.	Anderson, 1992
Bonamia exitiosa	*Ostrea chilensis*	New Zealand, Chile, Australia, North Carolina and Argentina	Intrahaemocytic protozoan parasite, infections quickly become systemic and are associated with high prevalences in austral Autumn. Susceptibility is thought to increase with environmental stressors including extreme temperatures, salinity, starvation, handling or concurrent heavy infection with apicomplexans. Studies of tank cohabitation indicate spread of parasite from infected individuals to neighbouring uninfected oysters	Berthe & Hine 2003 ; Hine 2002 ; Hine et al 2002
Bonamia roughlyi	*Saccostrea glomerata*	Australia	Systemic intracellular infections of haemocytes with the presence of focal abscesses in gill, connective and gonadal tissues as well as along the digestive tract. Disease process associated with low temperatures and high salinities	Mackin 1959; Smith et al 2000
Bonamia perspora	*Ostreola questris*	North Carolina	Single representative species of Bonamia that produces spores similar to those found in the Haplosporidians, typically low prevalences	Carnegie et al 2006
Perkinsosis, *P. marinus, P. olseni, P. quqwadi, P. cheasepeaki, P. andrewsi* and *P. mediterraneus*	Oysters and other bivalves, mussels show resistance to infection	Atlantic Coast of USA, Pacific Coast, Hawaii, Australia, and Mediterranean	Cause of mass mortality of *C. virginica* and severly impacted oyster culture in the eastern US. Causes systemic infections, with connective tissues harbouring trophozoites, mature trophozoites and tomont stages of the parasite. Ability to culture in the lab and direct transmission have provided experimental study of the activity and disease process of the parasites.	Vilallba et al 2004
Haplosporidium armoricana	*O. edulis* and *O. angasi* imported Olympia oysters (*O. conchaphila*)	France, Spain, and the Netherlands, Oregan	Spore masses occur in the connective tissues. Within an established culture setting the disease occurs at very low prevalence (1%) but when oysters from an unexposed population are introduced they are greatly impacted by the parasite	Bougrier et al. 1986; Azevedo 1999
Haplosporidians	Hatcheries of the pearl oyster *P. maxima*	Australia	Those harbouring infection were destroyed so impact of the parasite and strict identification of species is unknown. The parasite was noted to occur in the connective tissue of the digestive gland of those individuals identified as infected	Jones and Creeper 2006; Hine and Thorne 1998

Parasite	Host	Distribution	Impact	Reference
***Haplosporidium* sp.**	*Saccostrea cucullata*	Australia	Epizootics reaching 3-27% prevalence in the northeast. Infections were oftentimes heavy with plasmodia and sporulation taking place in the connective tissue, very little defensive response noted through histology (lack of haemocyte infiltration) but an abundance of brown cells were noted in these heavy infections	Hine and Thorne 2000, 2002
***Haplosporidium costale*, SSO**	*C. virginica*	Long Island Sound, New York to Cape Charles, Virginia, Also along the southern gulf of Saint Lawrence, the Atlantic coast, and within the Bras D'Ors lakes in Nova Scotia	Within its range along the US coast, it can be associated with seasonal mortality in late spring corresponding to the sporulation of the parasite (Couch and Rosenfield 1968, Andrews and Castagna 1978, Andrews 1984). The mode of transmission for H. costale is unknown and life cycle details including the presence or absence of intermediate host(s) is also unknown. Management of disease is sometimes facilitated through movement of stock to areas of low salinity where the disease process is impeded.	Couch & Rosenfield 1968; Andrews & Castagna 1978; Andrews 1984; Burreson & Ford 2004
Haplosporidium nelsoni, MSX	*Crassostrea virginica, Crassostrea gigas,* possibly *Ostrea conchaphila*	Along the Eastern coast of the US from Florida to Maine. In the Bras d'Or lakes Cape Breton Canada. Some reports from California, Washington, Oregon, British Columbia, France, Korea	Epizootics in localities in which disease is established reduce stocks by 90-95% Life cycle of the parasite is unknown, direct transmission has not been demonstrated. Plasmodia can be found systemically or focally within host tissues. Sporulation is sporadic in adults but often observed in juveniles. In the eastern United States high disease pressure in the spring follow mild winter temperatures and infection is limited by low salinity.	Andrews & Wood 1967; Ford & Haskin 1982; Chun 1972; Kern 1976; Kang 1980; Renault et al 2000; Freidman et al 1991; Freidman 1996; Mix & Sprague 1974; Stephenson et al 2003; Barber et al 1991; Burreson 1994; Burreson & Ford 2004

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Chapter 2

Comparison of *Haplosporidium nelsoni* infections of *Crassostrea virginica* across three localities in the Bras d'Or Lakes, Cape Breton, Nova Scotia.

2.1 Abstract

The unique environmental system of the Bras d'Or Lakes, Cape Breton, Nova Scotia Canada saw the appearance of the devastating pathogenic parasite, *Haplosporidium nelsoni,* within populations of the eastern oyster (*Crassostrea virginica*) in 2002 (Stephenson et al., 2003). Previously, this parasite had not been seen north of Maine, USA. The interrupted geographical progression of *H. nelsoni* northward along the western Atlantic coast along with the lack of evidence of spread of the disease outside of Cape Breton, suggests unique features amenable to disease expression within the Bras d'Or ecosystem. In 2005, sampling at three localities in the lakes set out to clarify the population at risk, as well as detect any low levels of pathogen in areas not exhibiting large scale mortalities at that time. The aim was to understand environmental and biological parameters for disease expression in the most Northerly reaches of the geographical range of *H. nelsoni.* The sampling sites included a population where MSX had been previously observed (Nyanza Bay), a site with no previous history of infection but adjacent to infected populations (East Bay) and a site at a remote location in the Bras d'Or Lakes where MSX had not previously been seen. Prevalences were 50% in Nyanza Bay, 30.7% in East Bay, and 28% in Lynche's River by PCR analysis. High prevalence indicated continued spread to areas previously determined to be free of the pathogen. Interestingly, histological analysis resulted in prevalences of 30% in Nyanza Bay, 23% in East Bay and 0% in Lynche's River. Intensities of infection at each OIE classified level were found, with sporulation taking place within two adult oysters collected from the index outbreak locality (Nyanza Bay). Prevalence differences seen in the two screening methods at one particular locality (Lynche's River) suggest the presence of *H. nelsoni* in this population without evidence of the development of active infections. The lack of histological evidence of MSX in Lynche's River may be due to population or environmental differences at this site relative to the other sampling locations

2.2 Introduction

The appearance of the protozoan parasite *Haplosporodium nelsoni* within the Eastern oyster, *Crassostrea virginica*, in the Bras d'Or Lakes in Cape Breton, Nova Scotia, Canada gives a unique opportunity to study the progression of disease within a newly infected population in a oceanic environment largely separated from the more typical coastal influences occurring in much of the parasite's range due to its shelter from the coastline.

Haplosporidium nelsoni is a protozoan parasite that infects several oyster species but is most detrimental in the eastern oyster, *Crassostrea virginica,* along the western seaboard of the Atlantic Ocean. It has continuously spread both northerly and southerly along this coast from its original locale in the Delaware and Chesapeake Bays reaching from Florida to Maine. This study set out to assess the prevalence of the parasite at several localities in the Bras d'Or Lakes and determine if low levels of disease existed in all areas in which the oyster hosts were found. This assessment utilized the two diagnostic methods available to identify and quantify infections with *H. nelsoni* within host tissues and allowed for comparison of these methods within the context of this new disease landscape. It also served to identify the population at risk and areas of disease that could be used to study disease criteria and extent of impact this parasite had in the region.

The eastern oyster, *Crassostrea virginica,* is an endemic species within the Bras d'Or lakes with an important ecological role within this environment. The overall production of the eastern oyster in Canada is concentrated within the eastern Maritime Provinces with western coastlines relying mostly upon the production of the Pacific oyster

(*Crassostrea gigas*). PEI has within the last twenty years been the leader in oyster production and export, but in recent years NB aquaculture has grown and has been producing choice export product as well (ACOA/DFO). NS oyster fishery is localized, due to tidal and environmental conditions, to certain sheltered coastline areas that allow for culture to occur. Within NS, a major area of culture has traditionally been within the Bras d'Or Lakes and their unique environmental landscape with low tidal pressures along with its typically warmer seasonal influences and shallow coastlines. Declines have steadily occurred within the Bras d'Or Lakes due to a number of factors and most recently can be attributed largely to disease and mortality caused by MSX. Large scale mortalities were first reported in 2002 by farmers who reported declines in stocks of over 95%. Testing was initiated and the disease agent was identified as *Haplosporidium nelsoni* also referred to as MSX (Stephenson 2003 et al.). The identification of this devastating parasite was the first report of an outbreak in Canadian waters. The parasite, despite its spread into northeastern US water systems, had not been noted through regular screening in Canadian oyster populations in Nova Scotia. Further testing confirmed that the parasite was contained within the lakes themselves and had not progressed along the Atlantic coastline of Nova Scotia (Stephenson 2003 et al.). The arrival of the parasite in a somewhat isolated water system has led debate as to the origin of its establishment. It also provides a unique opportunity to look at spread and impact within this system in order to gain insight about the disease. Regular screening was initiated after the index outbreak by the Department of Fisheries and Oceans (DFO) as well as mortality and impact studies by the Nova Scotia Department of Aquaculture and Fisheries. The unknown potential for the continued spread of this parasitic disease to other areas of

oyster culture in the Eastern Atlantic Provinces is of great concern to industry. Assessment of the disease within the Bras d'Or Lakes will lend insight into this parasite and disease impacts in its northernmost range.

In the fall of 2005, sampling was carried out in order to determine populations that may be harbouring infections at very low prevalences, which may be missed by standard screening methodologies. A typical sample used for screening includes sampling 60 oysters that if they are all negative provides one with 95% confidence that the prevalence in the population is less than 5%. However, sampling 300 oysters brings this 95% confidence to 1%, assuming 100% specificity and sensitivity of the diagnostic methods used. This was thought to provide insight into how the disease was spreading and also determine if there existed certain populations within the lakes who were able to maintain low levels of infection due to site specific environmental or biological influences. Two diagnostic methodologies, PCR and histology were used to determine prevalence at three sample sites in the lakes. Both methods are routinely used in assessing MSX infections. Molecular diagnosis using PCR acts to amplify a specific sequence in the parasite's genome. A positive PCR result indicates that the parasite was present within the tissues sampled. It does not, however, indicate a live parasite because parasite DNA could also be amplified from individual plasmodia that are dead or killed during the course of an immune response but still present in tissues or simply plasmodia within the water column at the time of sampling. A negative PCR result indicates no evidence of the parasite within the small portion of tissue used for analysis, but does not disprove the presence of the parasite elsewhere in the tissues and therefore is regarded as a tentative indication of the absence of infection. Microscopic diagnosis using histology allows for the

observation of the parasite in tissues sampled and any associated pathology if active infections are present. A positive histological identification can better determine if the parasite is active within the tissues while a negative result, similar to PCR negatives, may only indicate that plasmodia are not visible within the tissue section studied. The sampling localities were chosen according to previous screening information available at the time of collection, with Nyanza Bay having previously been identified as an established area of disease, East Bay having been screened in previous years with a single positive year but no associated mass mortality reported, and Lynche's River which had not been previously monitored.

2.3 Materials and Methods

2.3.1 Collection

Sampling took place in the fall of 2005 at three localities within the Bras d'Or Lakes, Nyanza Bay (N46º 02.270' W060 º53.240', depth: 1m, 9.5 ºC, 11.0 psu), Lynche's River (N45 º39.519' W060 º50.239', depth 1m, 10 ºC, 19.1 psu) and East Bay (N46 º 00.962' W060 º 23.453', depth 1m, 9.7 º C, 12.7psu) (Figure 1). Temperature and salinity were measured at each locality using an electronic sensor. Sampling was done through hand picking while SCUBA diving, only oysters of marketable size (76 mm) and above were collected. Sample sizes were determined with the intent of capturing very low prevalences by screening higher numbers of individuals at sites with no or little prior evidence of disease, however in the case of Lynche's River this was not possible due to a depletion of stock in this area as a result of recent overfishing. Sample sizes at each locality differed with n=120 collected from Nyanza Bay (historically positive site), n=58 collected from Lynche's River (no previous infection data), and n=300 from East Bay

(historically negative with a single positive result from screening carried out in 2003 by the federal Department of Fisheries and Oceans (DFO) and subsequent negative results from screening in 2004).

2.3.2 Oyster Processing

Individual oysters were washed free of fouling organisms upon collection, numbered, and the length and width of each measured and recorded. Using a shucking knife sterilized by submersion in 95% ethanol and subsequent flaming, oysters were shucked and their tissues removed from the shell onto a sterile bench covering. Sterilized forceps and scalpel were used to isolate three cross sections of mantle gill and digestive gland in succession, with the first section being placed in a labeled cassette and then into Davidson's fixative for histology; the second section being placed in a sterile 1.5 ml microcentrifuge tube containing 95% ethanol for molecular diagnostics; and the third section placed in a sterile 1.5 ml microcentrifuge tube in ice for biochemical analysis and then transferred to -80°C storage to avoid protein degradation. Remaining tissues were placed in sterile whirlpacks and stored on ice, and subsequently frozen at -80°C to preserve the remainder of each oyster.

2.3.3 PCR Diagnostics

DNA was extracted from tissue samples collected and stored in ethanol using a Qiagen DNeasy™ tissue extraction kit using the manufacturer's protocol. Total genomic DNA concentration was determined using a GeneQuant Pro spectrophotometer (Fisher) read at 260 nm and additionally measured ratios at 260/230 and 260/280 to ensure sample purity. Template DNA was added to a PCR reaction mixture containing 25 µl AmpliTaq Gold PCR Master Mix (AmpliTaq Gold DNA Polymerase 0.05 U/µl, GeneAmp PCR Gold

Buffer, (30 mM Tris/HCL, pH 8.05, 100 mM KCl) dNTP, 400 µm each, $MgCl_2$ 5 mM), 2.5 µl (1.0 µM) of each forward and reverse primer (OIE 2006, MSX-A (5'-GCATTAGGTTTCAGACC-3') and MSX-B (5'-ATGTGTTGGTGACGCTA-ACCG-3')), 10 µl (500 ng) Template DNA, and 15 µl molecular biology grade water. This mixture was then subjected to a temperature cycling protocol of initial denaturation of 94°C for 4 min, 35 cycles of 94°C for 30 sec, 59°C for 30 sec and 72°C for 1.5 min, and final extension at 72°C for 5 min (as per OIE diagnostic protocol for the detection of *H. nelsoni*) using a TECHNE TC-412 thermocycler (Fisher). Amplified DNA was electrophoresed on a 1% agarose gel containing 10 µl ethidium bromide adjacent to a molecular weight standard along with positive and negative controls and subsequently viewed under UV light using a Alpha Innotech imager (Fisher Scientific). Images were taken for each gel and annotated with sample numbers and amplicons of appropriate size (500 bp) compiled as positive diagnostic results for each sampling locality group.

2.3.4 Histology

Paraffin embedded blocks were prepared from tissues in Davidson's fixative and sections prepared according to the procedure as laid out in the OIE Manual for Diagnostic Tests for Aquatic Animals (2003) by the Nova Scotia Department of Aquaculture and Fisheries (NSDAF) in Truro, NS. Two-3 µm sections were stained with hematoxylin and eosin and read both at NSDAF (Truro) and Atlantic Veterinary College - University of Prince Edward Island. Screening initially for presence and absence of plasmodia and then assigning a subset of individuals based on the intensity rating system presented in the OIE Diagnostic Manual for Aquatic Animal Disease (2003) indicating R – Rare, L – Low, M – Medium, H – High, S – Systemic, and LO- Localized. Intensities encountered

within each of the sample localities were noted although the frequency of each intensity designation within these samples was not established. Any other signs of obvious pathology were noted for each location sampled.

2.3.5 Prevalence and Intensity Determination

From histological sections collected from individuals originating from each sample locality, prevalence (the percentage of individuals infected within a given sample locality) and intensity (the graded levels of parasites within a given infected individual) were determined. The presence of different parasite stages was also noted from histological sections (i.e. infective plasmodia versus sporulation within the tissues). From molecular diagnostic analyses, prevalence was determined for each of the sampling localities.

2.4 Results

2.4.1 Parasite Prevalence and Intensity

PCR analysis of mantle, gill and digestive gland resulted in the identification of *Haplosporidium nelsoni* infected oysters from all three sample localities (Figure 2). Prevalence by PCR was 50% (n=120) in Nyanza Bay, 28% in Lynche's River (n=58) and 31% in East Bay (n=300) (Table 1, Figure 3).

Histological sections of mantle, gill and digestive gland resulted in the identification of *Haplosporidium nelsoni* infected oysters from two of the three sample localities. It was noted through screening that oysters from Nyanza Bay were of poor overall condition, displaying opened and empty digestive tubules in the majority of individuals examined. Prevalence was 30% (n=120) in Nyanza Bay, 23%, prevalence in East Bay (n=300) and 0% in Lynche's River (n=58) (Table 2, Figure 3). None of the histologically positive

individuals were negative through PCR diagnostics. Observed intensities were also determined for Nyanza Bay and East Bay. Nyanza Bay had the greatest range of intensity classes from localized to heavy and with evidence of sporulation, East Bay showed evidence of typically light to moderate intensities (Table 3, Figure 3). Two individual oysters from Nyanza Bay also showed evidence of digestive gland sporulation of *H. nelsoni* (Figure 4).

2.5 Discussion and Conclusions

The results of this study provide evidence of a changing landscape of disease within the Bras d'Or Lakes system. The presence of typically high intensity infections at an overall prevalence of (50%) within the samples collected from Nyanza Bay lend support to the screening programs which have identified this area as having a well established disease pool within the lakes (DFO).

Active sporulation was found in the digestive glands of two oysters sampled from Nyanza Bay and although this was not the focus of this study it is of particular interest due to the fact that all oysters collected were of market size and typically sporulation is noted to occur most often in juvenile oysters within a population (Barber et al., 1991; Burreson, 1994). The poor overall condition of oysters sampled from Nyanza Bay could be a direct result of the MSX disease on the hosts or other contributing factors to the establishment of disease within this location, such as insufficient nutrition causing higher susceptibility to the impacts of the parasite.

The results of sampling conducted in East Bay are quite interesting in that they depict the impact of widespread disease within the lakes. This site had previously tested positive in histological screening in 2003 carried out by DFO and subsequently returned negative

results by both histology and molecular diagnostics in 2004 (DFO, personal communication). The sample size from this location in the present study was purposely large in order to investigate an area in which it was believed that low levels of disease were likely to exist. During MSX disease monitoring conducted by regulatory government laboratories, a typical sample size of 60 oysters is collected and subjected to both diagnostic methods (histology and PCR). In this study a sample of 300 oysters was collected from this locality in order to assess prevalence suspected to be below 5%. This approach would have allowed comparison in subsequent studies of protein expression from those oysters within a well established and sustained disease centre such as that seen to occur in Nyanza Bay with that of a population just newly infected or which has been better able to successfully deal with infection with a new pathogen. However, the results provided in this study indicated that the population suspected to have extremely low disease prevalence (East Bay), actually had developed into a population with prevalence comparable to that of the location of the initial disease outbreak (Nyanza Bay). This development coupled with the observation of a large multiyear class population of oysters at the time of sampling likely indicate the spread or amplification of disease into previously unaffected or limited affected areas of the lakes system. Despite the similar prevalences at these two localities, intensities observed within the infected oyster collected from East Bay harboured an overall lower intensity of infection than infected individuals from Nyanza Bay, which may suggest a role for multi year pressure in contributing to disease establishment in this system. A study investigating the protein profiles stored from these tissues compared to what would be seen at the same location today would provide interesting insight into whether or not the disease process differs

within a population largely encountering a pathogen at low levels as opposed to one who has had multi-year exposure to high levels of disease within the same area.

Samples collected from Lynche's River in 2005 provided the first evidence of MSX presence in this area, however it is worthwhile to note that the population found at this locality was surprisingly depleted either from over-fishing, poor overall conditions or lack of settlement in this location and did not fit the characteristically dense makeup of many of the populations typically impacted by the parasite. It should be noted that the depleted population may have been a result of poaching which was prevalent in the Bras d'Or Lakes immediately prior to the MSX outbreak. As this study was conducted only three years after the initial index outbreak, it is somewhat surprising that a small population such as that found at Lynche's River would show presence of the MSX parasite at all and raises questions about disease spread to this area.

Of particular importance when reviewing data from the Lynche's River is the absence of histological evidence of the parasite in oyster tissues from the individuals sampled. Despite that no *H. nelsoni* plasmodia were identified through histology, the PCR screening of the tissues collected from these same individuals indicated a prevalence of 28% from the study sample (n=58). While these two diagnostic methods have been reported to give differences when assessing prevalence (Burreson, 2000), these differences are thought to result from the increase in analytical sensitivity that PCR achieves. That study involved transplanting 400 uninfected oysters to lower York River and testing monthly using three diagnostic methods (PCR of haemolymph, PCR of tissues, and traditional histology), the methods reported differences in prevalence throughout the study (Burreson, 2000). PCR of haemolymph provided much higher

prevalences during the initial exposure and infection periods, while histology and tissue targeted PCR showed no evidence of *H. nelsoni.* Over much of that study period (mid-summer to December), prevalences reported for tissue PCR remained 10-30% higher then that reported for histological analysis. However, unlike the present study, plasmodia were detected in histological sections for all those data points in which tissue PCR positives were found (Burreson, 2000). The large discrepancy seen in the prevalence seen by PCR and histology from Lynche's River in the current study may be a reflection of the parasite's presence in this environment without development of active infections in these hosts. Samples were taken at the same time of year as other localities, so it is unlikely that these oysters will have progressed to a disease state. This finding suggests the presence of the parasite and associated pressure for infection development, without the actual establishment of disease. Oysters within the Lynche's River environment may be better able to resist infection or disease pressure. This could result from lessened stress, better overall initial health, or having access to richer environmental resources. Another limiting factor at this locality could be that environmental conditions are not conducive to disease development past the initial presence of organisms in tissues or spread of plasmodia to this environment, which contains limiting factors. Within the eastern US range of *H. nelsoni,* both salinity and temperature have been identified as impactful on the success of the parasite year to year, but when envrionmetnal conditions are not condusive to diseases infections are still detected within some members of the population though they tend to be of lower overall intensity and prevalence (Burreson and Ford, 2004). A difference in susceptibility has been noted for the parasite *Bonamia exitiosa* in *Ostrea chilensis* from New Zealand with environmental stressors including extreme

temperatures, salinity, starvation, handling, and concurrent heavy infections with apicomplexans found in association with an increase in host susceptibility (Hine and Thorne, 2002). In the current study, marked differences in temperature and salinity, however, were not found at the time of sampling between the three localities investigated in this study. It is unknown if significant differences in these two environmental factors occurred in the month's prior that may have had an impact on overall susceptibility of the three populations tested. The Bras d'Or Lakes do comprise a unique environmental setting with an almost entirely land locked salt and estuarine water system. There are low tidal influences (1m/s-03m/s, Dupont et al., 2003) on both the northwestern as well as the southwestern tips of Cape Breton Island, but due to the comparatively small connections these make with the coastal waters of the Atlantic, the flushing rates of the system are severely depressed and, when modeled, show a low rate of two years for the entire system (Petrie and Bugden, 2002). This low rate may account for some changes in dispersion of infective spores, and may explain why certain locations in the lakes are continued areas of established disease and others display changes over time.

Temperature and salinity for this environment are typically characterized by a layered system of low salinity and varied temperature found in surface waters, while the lower depth flowing waters are impacted by atmospheric pressure and the runoff of surrounding freshwater systems, and the deepest waters tend to exhibit higher salinities and more stable low temperatures (Parker et al., 2007). The seasonal fluctuations in temperature seen in the Bras d'Or Lakes range from 0°C in most parts of the Lakes in winter to a 6-10°C range in spring with a subsequent rise in summer and early fall to a high of 20°C in

the shallowest estuaries of the lakes (Parker et al., 2007). In spring, surface water salinities range from 20-30 psu depending on the locale of the lakes tested. Wright (1976) noted that heavy rainfall events had an impact on the salinity measured in a given area of up to 5 m in depth and much lower salinity levels within certain bays have also been noted near influxes of freshwater rivers (Davis and Browne, 1996). Seasonal barometric pressure has a significant impact on salinity in the lakes, with typically low salinity levels noted in rainy/runoff seasons of spring and fall and the more highly expressed salinities of the dry summer seasons. Young (1973) noted a marked fluctuation in salinities in Nyanza Bay over the course of a single day resulting from an August storm and associated high winds ranging from surface measurements of 4.3 psu one day to 8.4 psu the next and bottom salinity levels of 10.2 to 16.8 psu. Due to these large fluctuations the differences seen in susceptibility at any particular locale within the lakes could result from these traditional limitations of the parasite.

The combined characteristics of the Bras d'Or Lakes make them a unique and highly specified environment in which to support a unique biota. Without knowing the life cycle of this parasite, it remains unknown whether the absence of key intermediate host species may be impacting the establishment of disease in a particular bay. Many common species found along the Atlantic coast of Nova Scotia, are not found within the Lakes likely due to the lack of tidal pressures that many of the coastal forms are accustomed. The base of the food web within the lakes system are the planktonic communities, but these can often be depressed due to low nutrient levels found in many parts of the Lakes' system, although in some bays eutrophication does occur and provides

ample sources of nitrate. Along with phyto- and zooplankton many species of larval fish occupy the lakes including flounder, smelt, cod and mackerel (Parker et al., 2007). Benthic algal and seaweed communities thrive in certain portions of the Lakes and may be so plentiful as to disrupt the establishment of invertebrates in the benthos. Polycheates, mysids, foraminifera, mussels and sea urchins are also commonly found in certain locales in the Lakes. Groundfish and pelagic fish occur in the Lakes with key commercial species in both representative groups being cod and herring and several non commercial species including smelt, gaspereau, stickleback and eel (Lavoie, 1995). The role of the overall biotic communities may be an essential factor in spread and establishment on *H. nelsoni* within an environment, any key differences in the make up of these communities within the Lynche's River locality could contribute to the understanding of these impacts.

Certainly, the genetic influence on overall host susceptibility also exists (Gaffney and Bushek, 1996) and genotypes within a particular population are under some influences of the environment in which they live (Bushek and Allen, 1996). Oyster populations within the Bras d'Or Lakes have been characterized as genetically homogeneous (Vercaemer et al., 2010) and much movement of oysters has occurred around the Lakes thus; resistance and tolerance factors within the genotypes of this population are unlikely to play a role in the differences in prevalences noted among the populations studied. Regardless of the particular factors involved, this work identifies Lynche's River as a study locality with an interesting host population which could be investigated further with regards to host parasite interactions as well as the role of environment in disease establishment.

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Table 1: Prevalence determined from each sampling locality through PCR analysis. Numbers in brackets indicate number of oysters sampled and analyzed.

Sampling Locality	Prevalence Through PCR Analysis (n)
Nyanza Bay (historically positive)	50% (120)
East Bay (Some history of low level infection)	30.7% (300)
Lynche's River (no record of infection)	28% (58)

Table 2: Prevalence determined from each sampling locality through histological analysis. Numbers in brackets indicate number of oysters sampled and analyzed.

Sampling Locality	Prevalence Through Histological Analysis (n)
Nyanza Bay (historically positive)	30% (120)
East Bay (Some history of low level infection)	23% (300)
Lynche's River (no record of infection)	0% (58)

Table 3: Intensity ranges determined from each sampling locality through histological analysis. LO – localized, R – rare, L – low, M – moderate, H – heavy, S – systemic.

Sampling Locality	Intensity Classes Through Histological Analysis
Nyanza Bay (historically positive)	LO, R, L, M, H, S
East Bay (Some history of low level infection)	R, L, M
Lynche's River (no record of infection)	None

Figure 1: Bras d'Or Lakes Cape Breton, Nova Scotia, Canada. Sampling localities, active disease area; Nyanza Bay (1), limited disease identified; East Bay (2), new sampling locality; Lynche's River (3).

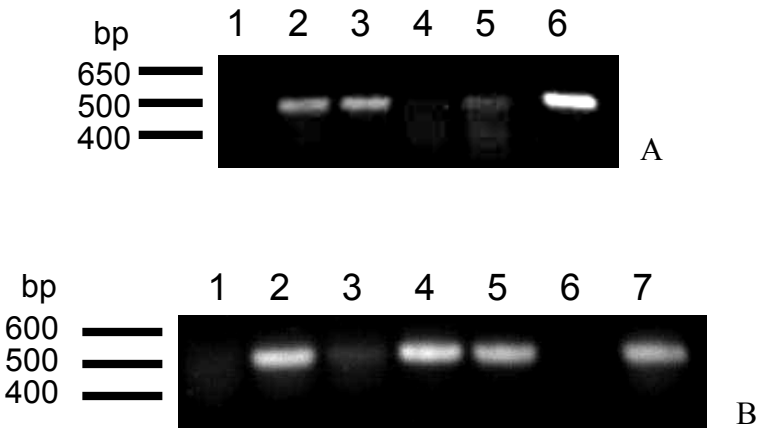

Figure 2: A. PCR results from four individuals (2, 3, 4, 5) collected from Lynche's River, NS, whose histological analysis showed no evidence of infection with *Haplosporidium nelsoni*, with positive (6) and negative (1) controls. B. PCR results from five individuals (1, 2, 3, 4, 5) collected from East Bay, NS, whose individual histological analysis showed no evidence of infection with *Haplosporidium nelsoni*, with positive (7) and negative (6) controls.

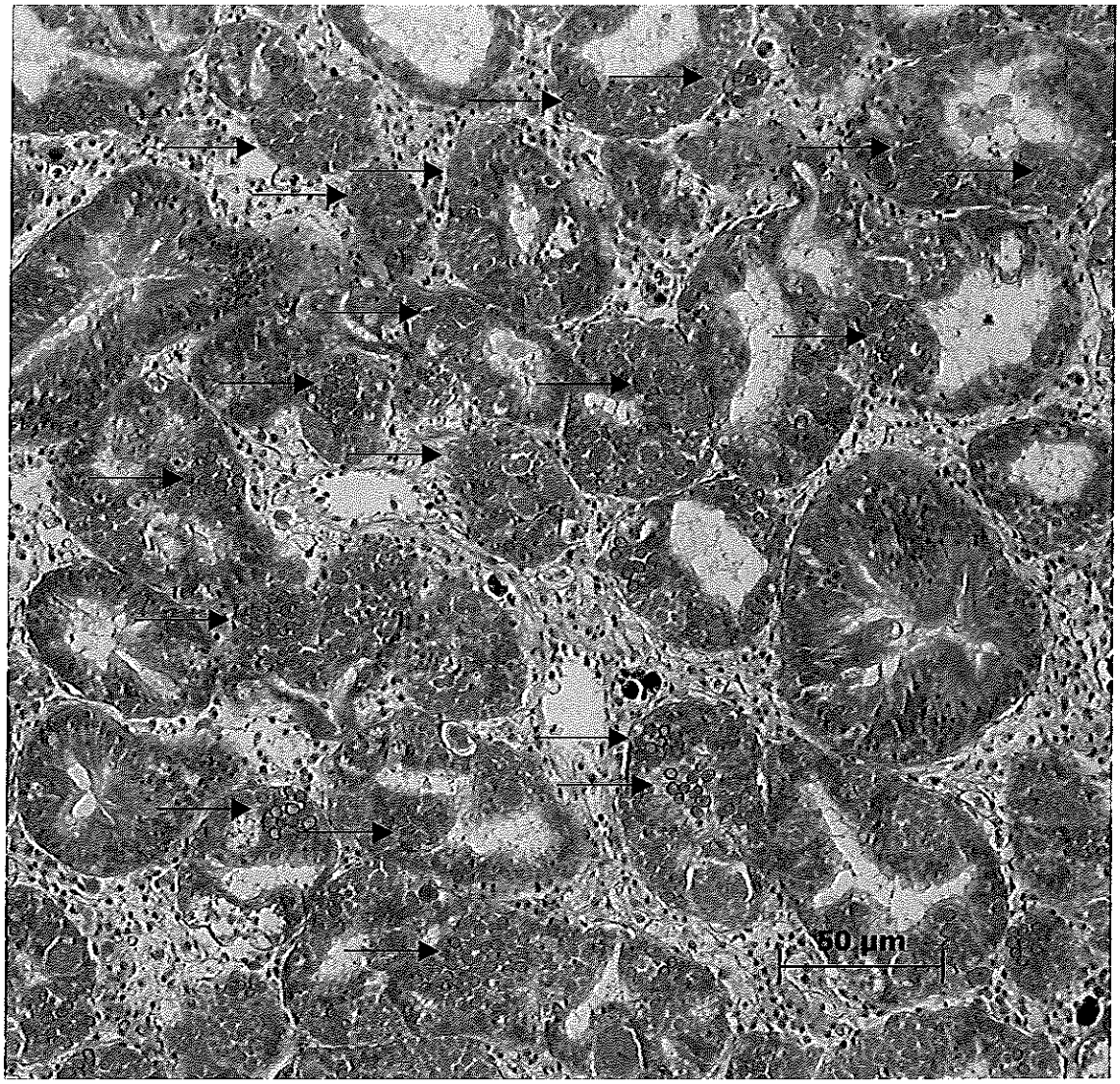

Figure 3: Evidence of active sporulation (→) within the digestive gland of *C. virginica* collected from Nyanza Bay.

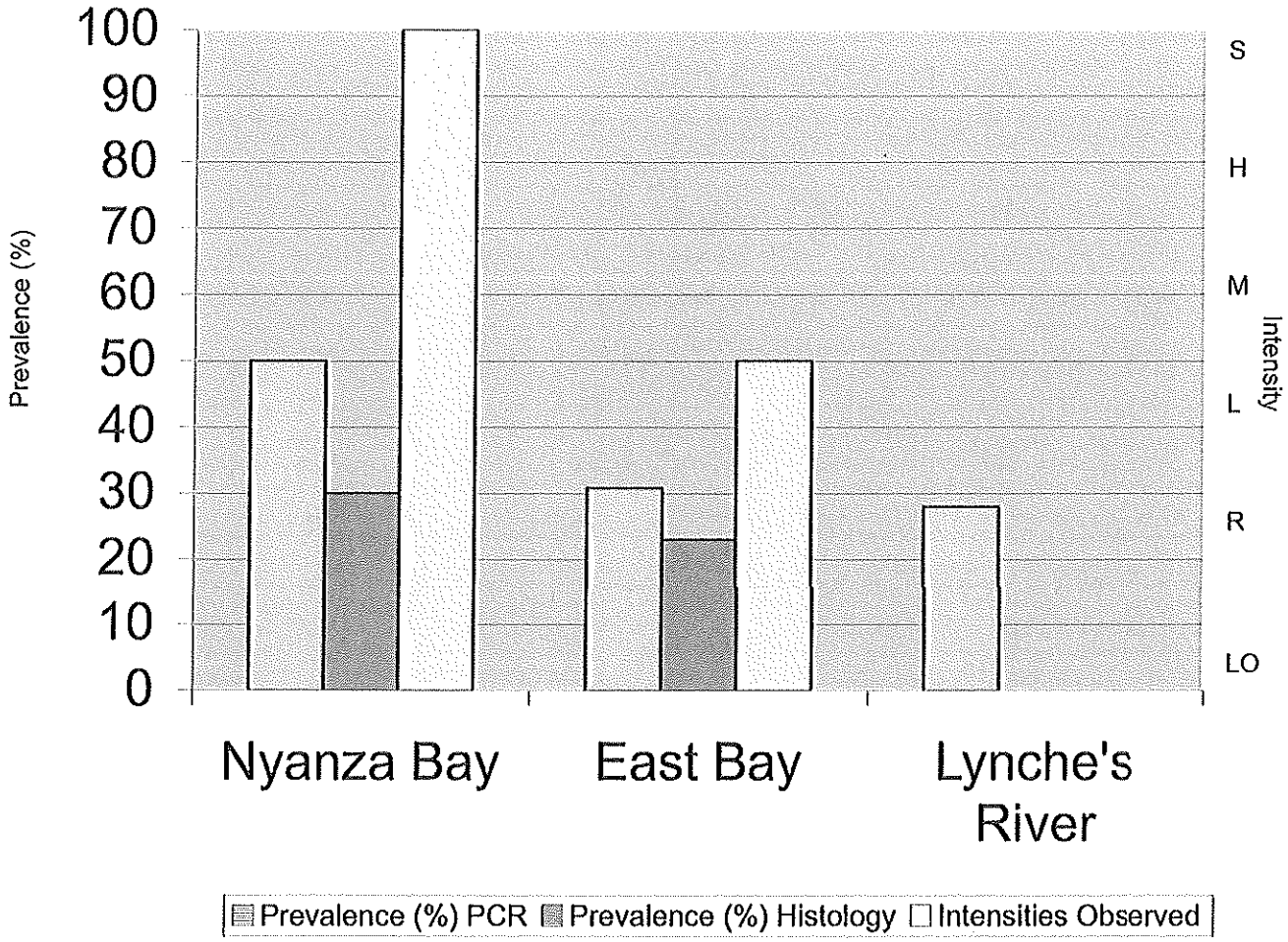

Figure 4: Prevalence (%) obtained using each diagnostic method (PCR and Histology) as well as intensities observed through histological screening from each sample locality.

Chapter 3:
Comparison of protein profiles of *Haplosporidium nelsoni* infected and uninfected *Crassostrea virginica* tissues collected from the Bras d'Or Lakes, Cape Breton, Nova Scotia.

3.1 Abstract

Study of the MSX parasite, *Haplosporidium nelsoni,* relies on *in situ* identification and analysis of the parasite in infected Eastern oysters, *Crassostrea virginica,* as the life cycle of this parasite is unknown and transmission and culture within the laboratory are not possible. Thus, information regarding the cellular activity facilitating the successful establishment and progression of the disease caused by *H. nelsoni* is limited. The arrival of this parasitic species within a new environmental system, the Bras d'Or Lakes, Cape Breton, NS, Canada, provided a new study system in which to investigate the disease caused by MSX infection. Tissues from individual oyster hosts, having been screened in Chapter 2, through two diagnostic methodologies were assigned to a disease state group for analysis: infected (high intensity systemic infections) or uninfected (no evidence of infection apparent through either diagnostic test). Constituent proteins from tissue collections of these two disease state groups were compared using one and two dimensional gel electrophoresis with the incorporation of differential detergent separation in order to identify any consistent protein changes associated with disease. Individual variability, as well as protein abundance among the several tissues studied presented difficulties in elucidating consistent differences in protein profiles between disease state groups. The methodologies presented, however, may provide new ways to study the MSX parasite despite traditional challenges.

3.2 Introduction

Haplosporidium nelsoni was first identified in Canadian waters in 2002 in populations of *C. virginica* from the Bras d'Or Lakes in Cape Breton, Nova Scotia, Canada (Stephenson et al 2003), a unique saltwater system with very low tidal pressures and low turnover rates due to their being almost completely landlocked (Dupont et al., 2003). Mortalities were reported in 95% of stocks affected and surveillance in subsequent years indicated some localized spread, however there were regions in the of the lakes where the presence of the parasite through PCR and histological testing (DFO- pers. com.) was not identified. The appearance of the parasite in the Bras d'Or Lakes was somewhat puzzling due to the northern spread of the parasite being interrupted (its range until that point had included the bulk of the eastern seaboard of the United States) (Burreson and Ford, 2004). From the established range along the coast of Maine there have been no reports of the parasite along the Atlantic coast of Nova Scotia. The parasite still eludes intense scientific investigation in that its lifecycle is as yet unknown and it is also not clear what, if any, intermediate hosts it employs during its spread. It is also not culturable, so investigators must rely on samples obtained through field infections. What is known about this parasite is its propensity to decimate naïve populations of the eastern oyster, when it is introduced (Andrews, 1968; Andrews and Wood, 1967; Ford and Haskin, 1982; Renault et al., 2000; Stephenson et al., 2003). Because of this, an early indicator as a flagship warning of spread and establishment would be of great use to those areas and parties who have yet to encounter this disease. Within Eastern oyster (*Crassostrea virginica*) culture practices, the ability to assess health of a stock in areas of MSX (*Haplosporidium nelsoni*) disease pressures, relating to acquisition of infection or infection intensity,

without having to carry out specific diagnostics back at a remote laboratory would be a valuable tool. The role of protein interactions involved in parasitic infections is at the forefront of parasite infiltration and success.

Parasite infections are among some of the most complex biological interactions undertaken between species (Combes, 2001). At the heart of this is the intimacy with which a parasite interacts with its host, and in particular within the protozoal parasites their impact on cellular defense and function has been well documented (Goedken et al., 2005; Montes et al., 1996; Stafford et al., 2002; Rollinghoff et al., 2001).

Protein interactions from both host cells and the parasite itself are often at the forefront of invasion, infection and proliferation (Shallom et al., 1999; Ayub et al., 2005). This can be seen in complex terms in the interplay of adaptive immune functions of many vertebrates. While innate immune functions are sometimes considered less complex, there are nevertheless comprised of a myriad of functional protein defense mechanisms, as well as many important protein functions utilized by parasites themselves to aid in their success at each stage of their lifecycle (Mosser and Brittingham, 1997; Locksley, 1997; Sacks and Sher, 2002; Turner, 2002).

Examination of protein profiles to map and analyze disease progression can be used to indicate possible proteins involved in both parasite establishment as well as host defense. Proteins exist within every structure and functional role within individual cells, and may not be readily accessed for study if targeted through a single extraction method. The isolation of proteins of different hydrophobicities from tissues is thus an important consideration when undertaking a proteomics study. Constituent tissue proteins are released through the paired action of mechanical disruption along with the presence of

buffers used to solubilize target protein groups. The myriad of proteins with regard to solubility, pH range and tolerance require the use of several specific buffers sequentially in order to retrieve the highest numbers of representative proteins from a given tissue. Ramsby (1999) developed a differential detergent fractionation (DDF) methodology targeted at doing just this in order to separate proteins occurring in different structural and functional compartments for study. Initially developed for use within cultured cell lines, the protocol has the advantage of lending insights in to the particular cellular compartment the protein changes identified may exist. Its simplicity and reduction of overall complexity in given samples lends itself nicely to use with both SDS-PAGE and two-dimensional gel electrophoresis (2-DGE) approaches. While SDS-PAGE separates proteins based on size and several samples can be run on the same gel, the 2-DGE approach separates proteins based on both isolelectric point and size thus allowing for more effective separation of individual proteins.

Due to the constraint involved in the study of MSX, an un-culturable parasitic species whose lifecycle is unknown, this study set out to use a proteomics approach to identify possible protein targets involved in parasite establishment and host defense by comparing extracts of infected and non-infected oysters via protein gel electrophoresis. Pre-fractionation was carried out in order to reduce the protein profile complexity and maximize the chances of identifying differences between study groups.

3.3 Materials and Methods

3.3.1 Oyster Collection

Oysters were collected from three localities in the Bras d'Or Lakes water system by hand picking while diving in the Fall of 2005. The three localities sampled were Nyanza Bay,

where the initial outbreak and sustained disease and mortalities have been reported; East Bay, with some past evidence of the presence of the parasite but no documentation of disease; and Lynche's River, a previously untested locality. The oysters from each group were tested for presence of the parasite through both histology and PCR and selected based on these diagnostic methods for analysis.

3.3.2 Selection Criteria for Protein Analysis

Each oyster was selected for analysis based on diagnostic screening method results. The negative oyster group (n=7) was established based on both histological and PCR analyses indicating the absence of parasite DNA and plasmodia. The positive oyster group (n=6) was selected based on individuals testing positive in PCR analysis, as well as showing high numbers of plasmodia in the tissues when histological sections were examined, with no evidence of sporulation. The two groups were therefore at opposite extremes of the disease spectrum with no samples representing low-level or mid-range infections. This was done in order to maximize differences between the study groups when assessing protein profiles.

3.3.3 Tissue preparation

Cross sections of digestive gland, gill and mantle tissues were excised from sampled oysters and frozen at -80°C. Tissues from those individuals selected from each population grouping, were homogenized on ice in 1.6 ml microtubes using a sterilized manual tissue homogenizer in the presence of lysis buffer (20 mM TrisHCl, pH 7.5) and protease inhibitors (1 mM PMSF and 50 mM EDTA). Following tissue lysis, samples were centrifuged at 4°C at 15000 x g for 10 min. The supernatant (20 mM TrisHCl, pH 7.5 buffer) was transferred to a new 1.6ml microtube for analysis and the pellet returned

to -80°C for storage. An aliquot of sample was assessed for protein concentration using a dye-binding assay with bovine γ-globulin as reference (Bradford, 1976).

3.3.4 Differential Detergent Fractionation

A differential detergent fractionation (DDF) method (Ramsby, 1999) was also applied to tissue samples to further separate constituent proteins so as to reduce the number and complexity of proteins in each fractions and more clearly elucidate differences in protein profiles between disease groupings. Tissue cell pellets from the TrisHCL extractions above were suspended in ice cold Buffer-A (300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 5 mM EDTA, 2 mM PMSF, ROCHE incomplete cocktail protease inhibitor in 10 mM PIPES, pH 7.2) containing 0.02% digitonin for 10 min and centrifuged at 1,000 × g. The resultant supernatant included primarily cytosolic proteins (Digitonin buffer). The pellet was then extracted with ice-cold 1.0 % (v/v) Triton X-100 in buffer-A for 30 min and centrifuged at 5,000 × g for 30 min. The supernatant obtained from this second buffer extraction represented the solubilized membrane fraction (Triton X-100 buffer). The remaining pellet was suspended in Buffer-A containing 0.5% deoxycholate, 1.0% Tween-40 and homogenized in a Teflon homogenizer and centrifuged at 7,000 × g for 10 min. The solubilized supernatant from the third extraction buffer was the nuclear fraction (Tween/Deoxycholate buffer). Finally, the detergent-resistant pellet was dissolved in 5% (w/v) SDS in 10 mM sodium phosphate pH 7.4, and contained the cytoskeletal fraction. The resultant extracts were assessed for protein concentration using a dye-binding assay (Bradford, 1976).

3.3.5 SDS-PAGE

Samples (5 µg) from the same individuals using all preparative detergent extraction buffers were prepared 1:1 in 2X SDS-PAGE sample buffer (2% SDS, 10% glycerol, 62 mM Tris-HCl, pH 6.8, 100 mM DL-dithiothreitol (DTT)) and run on a 12% SDS-polyacrylamide gel (Laemmli 1970) at 150V for 1.25 h. A BIORAD Prestained Broad Range SDS PAGE Marker was used as a molecular mass indicator Gels were silver stained (Swain and Ross, 1995) and images acquired with BioRad GS-800 Densitometer and compared visually through overlay of scanned samples.

3.3.6 2-D Electrophoresis

Individual samples were chosen for further analysis using 2-D gel electrophoresis (2-DGE) based on the same categories of disease from the populations studied as outlined above. Stored protein extract samples were transferred to dialysis tubing (3500 MWCO) and dialyzed for 24 h against 10 mM Tris-HCl pH 8.0. Following dialysis, samples were centrifuged (4ºC at 200 x g for 20 min) and the supernatant removed and frozen (-80ºC) until selected for analysis. Samples containing 200 µg of total protein were diluted 1:2 with rehydration buffer (6 M urea, 2 M thiourea, 2% CHAPS) containing 20 mM dithiothreitol (DTT) and 0.5% carrier ampholytes and added to 18 cm Immobiline dry strips pH 4–10 (Amersham-Pharmacia, Uppsala, Sweden). Isoelectric focusing in the first dimension was obtained using a Multiphor II system (Amersham Pharmacia) run at 30 V for 10 h, then increased to 8000 V over a 6 h period and held at 8000 V for 3 h. The sample strips were initially equilibrated for 15 min in 50 mM TrisHCl pH 8.8, 6 M urea, 30% v/v glycerol, 2% SDS, 65 mM DTT, followed by 15 min in 50 mM TrisHCl pH 8.8, 6 M urea, 30% v/v glycerol, 2% SDS, 135 mM iodoacetamide. For the second dimension,

strips were then laid upon and run in a 1 mm thick, 14% SDS-PAGE gel for 2100 Vh. Precision standards (Bio-Rad,161-0318) were employed in the second dimension gel to aid in estimation of protein molecular mass. Gels were then silver stained (Swain and Ross, 1995) and imaged in the GS800 BioRad densitometer for visual overlay comparison of profiles. Scans were compared visually initially for gross changes in the form of presence or absence of protein spots between infected and uninfected individuals subsequently more subtle changes were compared through numbering protein spots and determining the presence of spot intensity changes relating to infection. Variation was determined to be so high upon initial examination that further statistical analysis of band intensity was not performed.

3.4 Results

3.4.1 Differential Detergent Fractionation and SDS-PAGE

Whole tissue homogenates and extracts separated on SDS-PAGE gels showed a diverse number of proteins (Fig. 1). Consistent and intense protein bands were observed irrespective of disease state in the 20 mM TrisHCl, pH 7.5 buffer extract occurring at approximately 40-45 kDa, 30 kDa, 22 kDa, 17 kDa and 10 kDa (Fig. 1A). High molecular weight proteins were observed as highly variable and occurred in abundance in the range of 75-250 kDa (Fig. 1A). Through comparison of individual samples representing infected and uninfected oyster tissues, variation was observed in both groups, with variants also being noted among any given individuals within disease groups (Fig. 1A).

Incorporation of the differential detergent fractionation methodology allowed for separation of proteins from tissue homogenates. The proteins obtained from this protocol

were only able to be assessed through one dimensional SDS-PAGE due to the low sample volumes achieved through sequential fractionation of individual samples. Due to the amount of variability seen among samples run using SDS-PAGE, pooling of samples was not considered an appropriate approach in order to increase protein sample volumes. The protein profiles were more easily analyzed following extraction with each detergent buffer (Fig 1B to D). Protein recovery for each fraction was noted as high with numerous faint and intense banding patterns achieved for each. As seen in Fig. 1, consistent pattern of bands was observed among all samples within each of the extraction buffer extracts; for example the cytosolic compartment (Fig. 1B, 150 kDa, 45 kDa, 35 kDa and 22 kDa), the membrane compartment (Fig. 1C; 150 kDa, 90 kDa, 45 kDa, 35 kDa, 30 kDa) and nuclear compartment (Fig. 1D; 45 kDa, 40 kDa, 37 kDa, 30 kDa, 25 kDa). Variability was highest within the high molecular weight proteins with an abundance of bands concentrated in this area for most samples of all three pre-fractionation compartments. With the SDS-PAGE, variation among disease groups showed no consistent changes that could not also be attributed to individual variation within groups (Fig. 1).

3.4.2 2-D Gel Electrophoresis

Two-dimensional gel electrophoresis (2-DE) was also tested to resolve the complex protein matrix in the tissues and hopefully, better elucidate constituent proteins between diseased and non-diseased oysters (Fig. 2). Good separation and staining were achieved after incorporation of dialysis in preparative protocol (Fig. 2). Collections of consistent protein spot patterns were observed irrespective of disease group at 45 kDa and 30 kDa, with a wide spectrum of protein spots noted between these groupings on the individual gels. These profiles varied greatly among and between disease groups. High

molecular weight proteins were sparse in appearance on 2-DGE gels, with only two protein spots consistently observed among all samples located at approximately 75 kDa. A grouping of variable protein spots in the low molecular weight range indicated more basic proteins in the tissue samples run. Arrangements of three proteins within this range (25-27 kDa) were consistent among all samples. As with the one dimensional approaches, the variability within these tissues was similarly distributed among disease groupings and varied widely within individuals of each group (Fig. 2).

3.5 Discussion and Conclusions

It was hypothesized that the most heavily infected individuals (those chosen for analysis) in comparison with those who displayed no evidence of infection through both diagnostic methods, would have a discernable protein profile difference due to the impact of the presence of the parasites alone. Unfortunately, analyzing tissue homogenates by SDS-PAGE, as well as DDF extracted proteins, no discernable differences were identified due to the variability seen among all samples. The variability seen among infected and uninfected groups was similar to that seen within individuals from each of these groups. A larger sample size may have aided in reducing the impacts of individual variability among the oyster populations investigated. This lack of identifiable protein markers for the heavily diseased and non-diseased states may have missed more active immune protein reactions in that heavily diseased animals may have had to divert energy to basic physiological survival at a certain infection level, perhaps the study of different intensity MSX disease states may have been more informative. Individual variability is something that is quite often identified as impacting molluscan research (Huffman and Tripp, 1982; Auffret, 1985; McCormick-Ray and Howard, 1991; Chu and La Peyre, 1993; Ford et al.,

1994; Oliver and Fisher, 1995, Bayne 1998). There were several factors impeding more direct comparison in this study. The lack of control over timing of infection, disease progression, environmental conditions and health of individual oysters may have contributed to the variability observed. Thus, the active periods of infiltration and defense may have been missed. The tissues collected for protein analysis were targeted as being the sites at which the parasite would most likely be found in abundance. However, the combination of three complex tissues (digestive gland, gill and mantle) for analysis likely also contributed greatly to the overwhelming variability seen in this study. Host tissues should have most likely be dissected into distinct mantle, digestive gland and gill fractions, homogenized and run separately to obtain more homogeneous protein profiles. This would aid greatly in reducing variability, as well as provide localized information on those proteins expressed in these tissues under differing disease states. More localized dissection of actively infected tissues to obtain higher concentration of parasite proteins relative to host tissue proteins may assist in increasing the potential to observe proteins involved in that host-parasite interaction. However, given that *H. nelsoni* is a microscopic parasite and selective stains are required to distinguish it from host tissue histologically, it would be difficult to selectively enrich tissue extracts with parasite cells.

When assessing the host parasite interaction of a system in which so little is known about the parasite in question, protein targets have the potential to yield a wealth of information regarding the actual cellular processes underway. While research surrounding MSX in particular has been fraught with difficulties, targets for protein analysis have looked primarily at overall protein concentration, hematological proteins, and host defense protein identification (Feng 1970; Ford 1986; Barber 1988). However, the use of

proteomics in studying the subtle effects of pollutants has steadily grown with the development of more precise technologies such as 2-DGE and more recently DIGE. In their review of these applications relating to a proteomics study investigating oxidative stress in bivalves Sheenan and McDonagh (2008) highlight the importance of physiological traits of comparative groups as well. In the analysis of the bivalve QX disease in the Sydney rock oyster, caused by the paramyxean parasite *Marteilia sydneyi*, *Saccostrea glomerata,* protein markers were successfully identified using the 2-DGE approach targeting both resistant and susceptible oyster tissue groups as well as the sporulation stage of the parasite in haemolymph for comparison (Simonian et al., 2009 a, b)

The environment from which samples were taken for the current study was a relatively new area of expression for MSX disease, and while samples were taken from different locations in order to make comparisons between environments of intense and low disease pressure the progression of disease at each locality sampled resulted in similar prevalences occurring from each location (Chapter 2). In addition, the comparison of samples across sampling localities may result in differences purely resulting from environmental differences found at each locality (food, temperature and pH regime, stressors, predation). While the Bras d'Or Lakes are quite homogenous and exposed to many of the same environmental pressures, the impacts of each of these even expressed as slight variances at each location was not known.

Another factor which may have been at play is the limits of the diagnostic tests available. While a negative result by both PCR and histology is the best indicator of absence of the parasite, there still remains the possibility of the parasites' presence not being captured by

either technique and instead being a more localized focal infection contained within adjacent untested tissues. This would likely have an impact on protein expression and results in similar profiles being observed for both an established positive and a presumptive (though false) negative sample. Similarly, it is not known what difference, if any, would exist in protein profiles obtained from a sample infected with one plasmodia versus many, or even if a sample contained unviable or killed parasites. Both the differential detergent fractionation as well as the 2D electrophoresis methodologies show promise in their separation and clear representation of constituent sample proteins. It was determined that dialysis of samples before analysis using 2D electrophoresis greatly impacts the clarity and isoelectric focusing of marine mollusk tissue samples. The additional detergent extractions used typically in cell culture to target different cellular fractions (Ramsby, 1999) greatly reduced the number of proteins run in SDS-PAGE for each sample, and the proteins proved more clearly separated and defined for each extract. It is not clear if the detergents separated proteins based on cellular organization (cytosolic, membrane, and nuclear fractions) as hypothesized with cultured cells or solely on lipophilicity, but this would be interesting to investigate and could be used to focus further study based on where defensive action is thought to occur in host-parasite systems such as MSX and *C. virginica*. Many of the high molecular weight proteins visualized on the SDS-PAGE runs of the DDF treated samples were not clearly separated using the initial homogenization extraction, nor were they abundant enough to be studied through 2-D gel electrophoresis. For these proteins, the DDF protocol showed a specific application in targeting these profiles from the tissues studied. Larger aggregated samples would be needed to obtain sufficient protein concentrations

for full 2-DE analysis, but in order to achieve an appropriate level of individual variability for comparison, another factor such as the use of a single tissue type would have to be substituted in the initial collection protocol.

Continued study of the MSX parasite, *Haplosporidium nelsoni* within the eastern oyster population of the Bras d'Or Lakes would need to isolate specific tissues and localities to target in order to implement the techniques developed here to investigate protein profiles relating to infection and disease in this host-parasite system.

3.6 References

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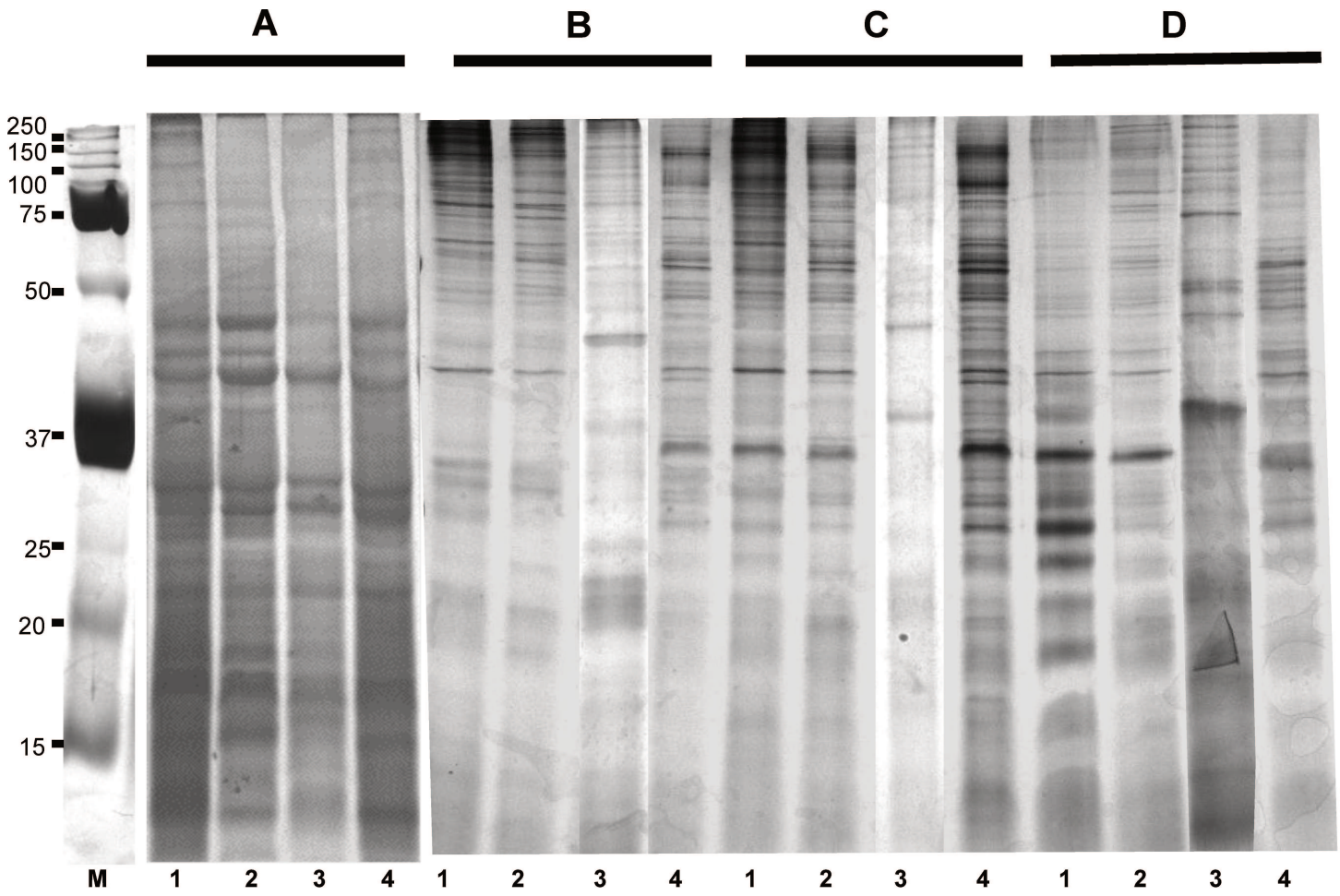
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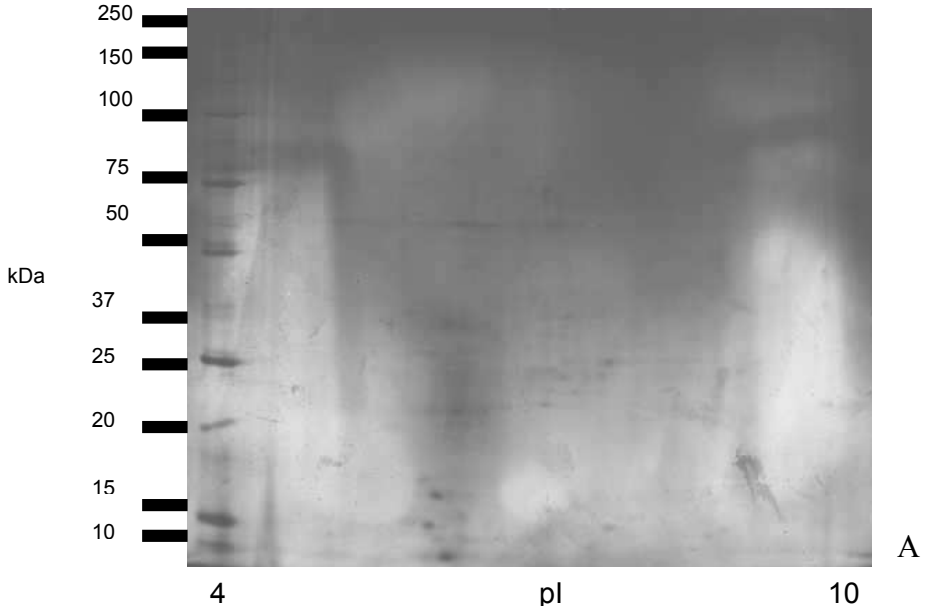
Figure 1: One dimensional SDS-PAGE profiles of gill, mantle, and digestive gland of four representative individual oysters with (1, 2) and without (3, 4) evidence of infection with *Haplosporidium nelsoni*. Different detergent fractions (DDFs) included, (A) 20 mM TrisHCl, pH 7.5 buffer; (B) Digitonin buffer; (C) Triton X-100 buffer; (D) Tween/Deoxycholate buffer all separated on 12% polyacrylamide gels.



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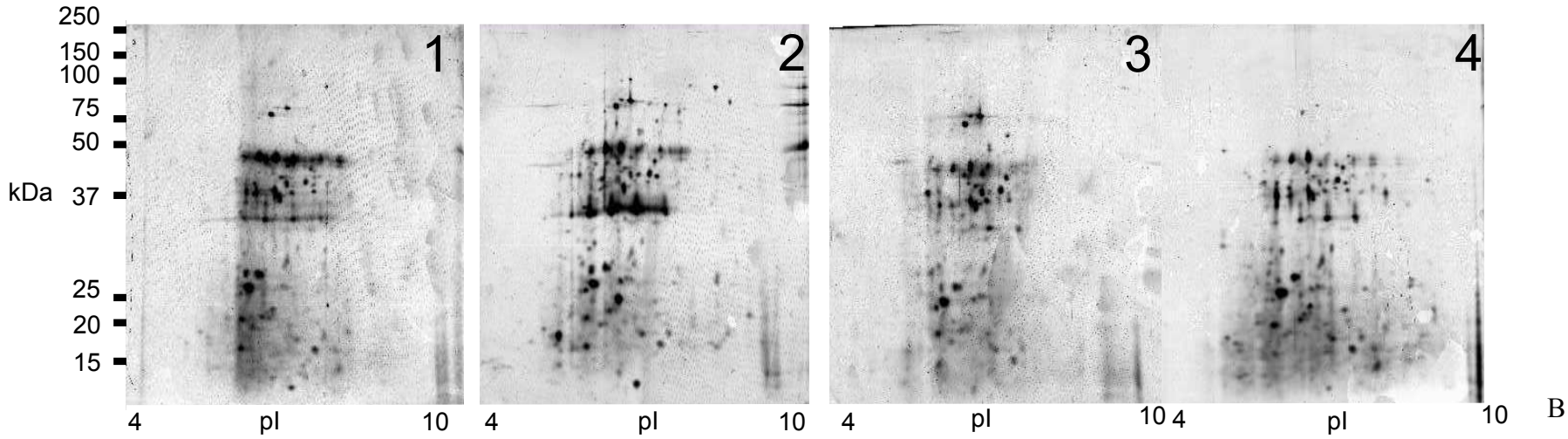

Figure 2: Two-dimensional gel electrophoresis profile of gill, mantle and digestive gland proteins for one sample before incorporation of a dialysis preparative protocol (A). Two-dimensional gel electrophoresis profiles of gill mantle and digestive gland proteins of four representative individual oysters (B) with (1, 2) and without (3, 4) evidence of infection with *Haplosporidium nelsoni*. First dimension run on pH 4-10 IPG strips and the second dimension run on 14% polyacrylamide gels.

Chapter 4

Differential protein expression from haemolymph of *Crassostrea virginica* following field infection with *Haplosporidium nelsoni* in Gloucester Point, Virginia USA.

4.1 Abstract

Study of the eastern oyster, *Crassostrea virginica,* proteome in the context of infection with *Haplosporidium nelsoni* has often proved difficult due to the individual variability encountered when undertaking comparisons of similarly treated individuals. In order to attempt to minimize this effect, a study was carried out within the Chesapeake Bay system of Virginia, USA, targeting haemolymph proteins of individual naïve oysters exposed to an MSX endemic area and sampled over time. Prevalence of infection with MSX, as determined by histology, in 2006 reached 57 % (100% by PCR) and with 79% associated mortality. In 2007, prevalence was 88.6% (100% by PCR) with only 21% mortality. The study population from 2007 displayed a range of infection intensities, which were group into four intensity classes (N-none, L-low, M-medium, H-high) for protein and enzyme analysis. Overall protein concentration differed significantly over time within those individuals who showed no evidence of infection by histology and among all intensity groupings at the final sample time, two months post-deployment. One dimensional gel electrophoresis of individual haemolymph samples identified a protein band of interest which was present most often in the samples collected after exposure to the MSX infected environment. Subsequent MS analysis of representative samples of this band identified actin as the most likely identity, and band presence was found most abundant at the two post-deployment sample times in light and medium intensity classes and saw a significant increase in abundance in the final sample of the high intensity class. Similarly, zymography analysis identified a series of high molecular weight protease bands (140-200kDa) found in samples collected pre-infection and occurring most often in those individuals that went on to develop high intensity infections. Lysozyme activity varied widely among intensities and across sample times while alkaline phosphatase activity increased significantly over time within low medium and high intensity class groupings. This approach to the study of susceptible individual hosts over the course of exposure and infection with *H. nelsoni* has the potential to provide new insight to this host parasite system.

4.2 Introduction

MSX along the Eastern Seaboard

Haplosporidium nelsoni, is thought to have first been introduced to the eastern United States through transfer of its natural host species, *Crassostrea gigas* in the 1950s from the west coast of the U.S.A. (Burresson and Ford, 2004). It was first detected in *Crassostrea virginica* in Delaware Bay in 1957, and within a few years had spread to Chesapeake Bay. Although the spread of MSX now encompasses the bulk of the eastern seaboard of the United States, it has been associated with disease and severe mortality in only those areas in which it appears that the correct environmental conditions are met for the parasite's proliferation. These include temperature and salinity, which seem to regulate the progression of disease. Ranges of 5-20°C have been suggested as the typical range in which disease occurs, likewise, a salinity of 15 psu is required for initial infection and a range of 18-20 psu is most likely to converge with high mortality and overall success of the parasite (Haskin and Ford, 1982; Burreson and Andrews, 1988). Parasite proliferation is highest at salinities above 20 psu (Andrews, 1968) and *H. nelsoni* does not survive in salinities below 10 psu (Ford, 1985; Ford and Haskin, 1988). The parasite is spore forming and is most commonly identified within oyster tissues in the stage of multi-nucleated plasmodia. It is thought to initially enter oysters of any age within the gill and mantle tissues and as the disease progresses it spread to other tissues, and can commonly be found in very intense infections in the digestive gland. The complete life cycle has not been characterized and it is unclear whether the parasite incorporates any or multiple intermediate hosts into its proliferation in any given estuary. The annual cycle of infection in the Chesapeake has been well documented with oysters first becoming

infected in early summer with associated mortalities peaking in late summer and early fall (Andrews, 1966; Ford, 1985; Burreson and Ford, 2004). In late fall prevalence and mortalities decrease with surviving oysters harbouring low level intensity infections that lead to a wave of fresh mortality in the spring. The annual cycling suggests a correlation of increased parasite abundance and resultant mortalities during summer drought conditions and subsequent decreases associated with spring freshets (Burreson and Andrews, 1988; Burreson and Ragone-Calvo, 1996).

Perkinsus marinus is another protozoal pathogen found in *Crassostrea virginica* in the Chesapeake (Mackin 1950). It is often found to occur following the infection wave of *Haplosporidium nelsoni*, with the parasite infecting oyster haemocytes from late summer into the fall. This parasite seems particularly adept at causing detrimental effects in the eastern oyster, while *Crassostrea gigas* and *Crassostrea ariakensis* seem to deal better with exposure and infection. There has been some evidence of specific *C. virginica* anti-proteolytic activity targeting parasite proteases in lab investigations (Faisal et al., 1999). It would appear that there are certain defenses occurring within the haemocytes and plasma that may indicate how and why certain infections are more successful than others. While *Haplosporidium nelsoni* once established in its host is found to occur within the tissues, previous protein studies have established that tissue analysis leads to a diverse array of proteins profiles with inherent individual variability. In contrast, studying Perkinsus and its effects through targeting the haemolymph has shown some promise in identifying proteins and activities of interest (Faisal et al., 1998; Oliver et al., 1999a,b, 2000; Chu et al., 1993, LaPeyre 1995, Chu and La Peyre, 1993a,b; Garreis et al., 1996). One measure used to study changes in constituent proteins found in biological samples is

protein electrophoresis. In one dimensional gel electrophoresis proteins are treated in order to unfold their native shape and are then separated based on size (molecular weight). With two-dimensional gel electrophoresis, proteins are first separated based on isoelectric point (pI) a point at which their net charge is zero in their natural structure and then further separated along molecular weight as in one dimensional gels (O'Farrell, 1975). Molecular weight is determined primarily by the amino acid sequence of the protein and the isoelectric point is determined both by the charge of the amino acids found in the protein as well as in the post translational modifications impacting overall net charge. Assessment of proteins in a sample using these methods provides a diverse profile allowing for the determination of the number of proteins as well as relative concentration of these in a sample (based on band intensity). These profiles can be a signature of a particular cell type or tissue and changes in this signature can be affected by disease, physiological stress , nutritional status, developmental stage, and environmental fluctuations.

As seen in *Perkinsus* sp. and *Urastoma cyprinae* (Brun et al., 2000) infections, proteases appear to play key roles in host parasite interactions. Proteases occur in all organisms breaking down proteins through hydrolysis of peptide bonds between amino acids in a polypeptide chain. Physiological functions include digestion of protein as food, to more regulated cascades effecting complement, apoptosis, blood clotting and the invertebrate prophenoloxidase activation (Morrissey, 1998; Cho, 2002). In response to physiological changes, proteases can trigger rapid shifts in metabolic and immune functions in within an organism.

Parasite derived proteolytic enzymes can aid in infection through penetration and digestion of host tissues (Sung and Dresden, 1986; McKerrow, 1987, 1989; Knox and Jones 1990; Berasain et al., 1997; Perkins et al., 1997) as well as serve to evade the actions of the host's immune response (Ellis et al., 1990; Kamata et al., 1995; Garreis et al., 1996).

Other enzymes also play key roles in the host-pathogen interaction. Within the innate immune system, lysozyme has been identified as a key enzyme that catalyzes the hydrolysis of 1, 4-beta-linkages shared between N-acetylmuramic and N-acetyl-D-glucosamine residues found in peptidoglycan of bacterial pathogens (Jolles, 1996). In marine species, lysozyme is measured mainly in serum or plasma reflecting the proinflammatory phagocyte response (Lie et al., 1989, Saurahb and Sahoo, 2008; Fange et al., 1976). Specifically in mollusks, lysozyme has been characterized from a number of species (Xue, et al., 2010). Details of specific action within a given species nor its optimal conditions (particularly the pH range of activity within these systems) have not been fully characterized.

Alkaline phosphatase has been described from many groups of organisms as an enzyme whose action removes phosphate groups from proteins and other molecules including nucleotides in basic environments (Crofton, 1982). The role of alkaline phosphatase in immune function is not clearly understood, though in a number of organisms and disease interactions its activity has been reported as altered in response to infection. In molluscan research, alkaline phosphatase activity has been studied in relation to disease,

chemical contaminants and natural physiological composition (Xue and Renault 2000; Faisal, 2000; Cima et al., 2000; Evtushenko et al., 1984)

In order to investigate protein interactions within this host parasite system and eliminate as much individual variability as possible, protein comparison of haemolymph from individual oysters at different time points would help identify protein changes related to infection over time. Oysters collected from Rappahanock River system, which is free of *Haplosporidium nelsoni* and displays only sporadic infections of *Perkinsus marinus,* were bled and subsequently released into the York river for field infection and then were used to track protein changes over time and with exposure to both parasites. Haemolymph collected after exposure and related to diagnostic determinants (prevalence and intensity data for each parasite and in each individual oyster) allowed variability to be controlled so that protein and enzyme differences to be more accurately studied. Although this study relies on field samples (allowing for dual infections as well as environmental factors to have an impact), it can provide insight into changes over time in individuals that may be involved in one or both of these devastating disease processes.

4.3 Materials and Methods

4.3.1 Sampling

Oysters (n=100) were collected over two sampling seasons from the Rappahannock River system using a mechanical dragging fork. Each oyster was washed and labeled with either nail polish number (2006) or numbers epoxied to their shells' (2007). Once labeled, each oyster was measured and their shells were notched using a circular saw so that an aliquot of haemolymph (1.0 ml) could be bled and put directly on ice. Bled

oysters were then deployed in vexar mesh bags (50 per bag) in the York River adjacent to the Virginia Institute of Marine Sciences' Gloucester Point research station, for field infection. In the 2006 field season oysters were deployed in May and subsequently retrieved for processing in August 2006. In the 2007 field season, oysters were deployed in May and retrieved in July 2007, with an additional aliquot of haemolymph collected two weeks post deployment, resulting in three haemolymph samples (1-naïve oysters, 2- two weeks after field deployment, and 3- two months after field deployment) collected from each individual oyster in this second year of field infection.

4.3.2 Oyster Processing

Upon collection each oyster was thoroughly washed free of fouling organisms and examined for obvious signs of morbidity. Those that were gaping and dead were separated and viable oysters were measured and put forward for processing. Oysters were re-notched using a circular saw and haemolymph bled from their cardial cavity. At this final sampling, all the haemolymph was drained from each individual and the oysters were shucked and tissues collected aseptically. Cross sections of mantle, gill and digestive gland were collected and placed in Davidson's fixative for histology, 100% ethanol for PCR and frozen at -80°C for protein analysis

4.3.3 Histology

Samples of digestive gland, gill and mantle collected from individual oysters were placed into labeled histology cassettes and preserved in Davidson's AFA for at least 24 hours. Once fixed, the tissues were subsequently embedded in paraffin, sectioned 5-6um and placed on slides and stained with hematoxylin and eosin (H&E). Stained slides were read by the histopathologist of the VIMS shellfish research laboratory and results of infection

(presence of any pathogens as well as intensity of MSX using OIE grading system), associated pathology, oyster sex and overall health were provided for analysis.

4.3.4 PCR Diagnostics

Tissue samples collected and stored in ethanol were subjected to DNA extraction using a Qiagen DNeasy tissue extraction kit and the manufacturer's protocol. Total genomic DNA concentration was assessed using a Fisher GeneQuant Pro spectrophotometer reading at 260 nm. Template DNA was added to a PCR reaction mixture as outlined in the OIE diagnostic manual for Aquatic Disease. Samples were then subjected to a temperature cycling protocol of initial denaturation of 94°C for 4 minutes, 35 cycles of 94°C for 30 seconds, 59°C for 30 seconds and 72°C for 1.5 minutes, and final extension at 72°C for 5 minutes (as per OIE diagnostic protocol for the detection of *H. nelsoni*) using a Fisher TECHNE TC-412 thermocycler. Amplified DNA was electrophoresed on a 1% agarose gel containing 10 µl ethidium bromide adjacent to a bp molecular weight standard and subsequently viewed under UV light using a Fisher Alpha Innotech imager. Images were taken for each gel and annotated with sample numbers and amplicons of appropriate size (200 bp) compiled as positive diagnostic results.

4.3.5 Prevalence and Intensity Determination

Prevalence for each field season's final harvest was calculated for both PCR and histological diagnostic analysis. Intensity of infection for each individual oyster was discerned through histology, using the OIE diagnostic manual for Aquatic Animal Diseases classifications including N – None, R – Rare, L – Low, L-M – Low-Medium, M – Medium, M-H – Medium High, H – High, S – Systemic, and LO- Localized.

4.3.6 Selection Criteria for Protein Analysis

Each oyster was placed into an intensity class and compared amoung all classes. Group one consisted of those individuals with a intensity class of no plasmodia observed (N), group two consisted of those individuals with intensity classifications of rare (R) and low (L), group three consisted of those individuals with intensity classifications of low-medium (L-M) and medium (M) and group four consisted of those individuals with intensity classifications of medium-high (M-H) and high (H). Nine individuals from each group were chosen at random to be run for protein profile comparison. Each individual chosen had three representative haemolymph samples for comparison, one taken before deployment (#-1), one taken two weeks post-deployment (#-2) and a third taken at the end of field infection period 2 months post-deployment (#-3). Differences were investigated both within an individual's haemolymph over time, as well as between each of the outlined intensity groups at any given sampling period.

4.3.7 Assessment of Protein Concentration

Haemolymph samples were thawed on ice and centrifuged at 15,000 x g for 10 minutes at 4°C to separate cells from serum. Haemolymph supernatant (serum) was collected from and aliquoted into 100 µl working stocks and returned to -80°C for storage. Protein concentration of each sample was determined using the Bradford protein assay (Bradford, 1976) by adding 5µl of haemolymph of each sample in triplicate to a 96 well plate. Diluted commercial Bradford reagent solution (250 µl BioRad protein assay dye reagent 1:4 in deionized water) was added to each well including a positive and negative control and the plate read immediately at 590 nm on a THERMOmax microplate reader. The resulting concentrations were calculated through comparison with a previously

established standard curve for the Bradford solution developed with dilutions (0.1-1.4 mg/mL) of bovine gammaglobulin (BioRad). The plate and resultant optical densities were analyzed using the program SoftMax Pro.

4.3.8 Two-dimensional gel electrophoresis

Individual haemolymph samples were selected for analysis using 2-D gel electrophoresis (2-DGE) based on the infection intensity and collection time as outlined above. Samples were transferred to dialysis tubing (3500 MWCO) and dialyzed for 24 h against 10 mM Tris-HCl pH 8.0. Following dialysis, samples were centrifuged (4ºC at 200 g for 20 min) and the supernatant removed and frozen (-80ºC) until selected for analysis. Samples containing 200 μg of total protein were diluted 1:2 with rehydration buffer (6 M urea, 2 M thiourea, 2% CHAPS) containing 20 mM dithiothreitol (DTT) and 0.5% carrier ampholytes and added to 18 cm Immobiline dry strips pH 4–10 (Amersham-Pharmacia, Uppsala, Sweden). Isoelectric focusing in the first dimension was obtained using a Multiphor II system (Amersham Pharmacia) run at 30 V for 10 h, then increased to 8000 V over a 6 h period and held at 8000 V for 3 h. The sample strips were initially equilibrated for 15 min in 50 mM TrisHCl pH 8.8, 6 M urea, 30% v/v glycerol, 2% SDS, 65 mM DTT, followed by 15 min in 50 mM TrisHCl pH 8.8, 6 M urea, 30% v/v glycerol, 2% SDS, 135 mM iodoacetamide. For the second dimension, strips were then laid upon and run in a 1 mm thick, 14% SDS-PAGE gel at 2100 Vh (116.7 V * 18 h). Precision standards (Bio-Rad,) were employed in the second dimension gel to aid in estimation of protein molecular mass. Gels were then silver stained (Swain and Ross, 1995) and imaged in the GS800 BioRad densitometer for comparison of profiles.

4.3.9 One-dimensional gel electrophoresis

A total of 5 µg (1:1 sample and 2X sample buffer solution 0.5 M Tris-HCl, pH 6.8, glycerol, 10% w/v SDS. 0.2% (w/v) Bromophenol Blue) of each representative sample was run on 10% polyacrylamide gels for 1.5 hours at 150V. Gels included samples from each intensity class at the three different time points. Gels were then silver stained (Swain and Ross 1995) adjusting the protocol to eliminate the use of glutaraldehyde in order to maintain protein bands suitable for use in Mass Spectrometry analysis. The resulting bands were viewed and scanned using an 800S BIO RAD densitometer imager.

4.3.10 Band Selection and Mass Spectrometry Analysis

Bands were assessed based on presence or absence between intensity groups or across time. A similarly sized protein band displaying consistent changes identified among the individuals were considered bands of interest and four representatives of this band were excised from the SDS-PAGE gels using a razor blade and aseptic conditions under a flow hood in order to minimize contamination of common environmental proteins and submitted for MS analysis.

4.3.11 MS Identification of protein changes

Excised proteins were placed in 1.5 mL microcentrifuge tubes, washed 3 times for 10 min with 100 µL of 50% acetonitrile (ACN) in 25 mM NH4HCO3 and dehydrated in 200 µL of 100% ACN for 10 min. Proteins were reduced with 200 µL of 10 mM DTT in 25 mM NH4HCO3 at 56 °C for 1 h, followed by alkylation in 200 µL of 25 mM NH4HCO3 containing 55 mM iodoacetamide darkness at room temperature for 45 min. The reduced and alkylated gel pieces were washed 2 times with 100 µL of 25 mM NH4HCO3 and 50% ACN containing 25 mM NH4HCO3 for 10 min. Gel pieces were dehydrated with

200 µL of 100% ACN for 20 min and residual ACN was removed in a Speedvac for 10 min. Gel pieces were rehydrated with 20 µL of 12.5 ng mL− 1 trypsin (Promega, Madison, WI, USA) in 25 mM NH4HCO3 and incubated at 37 °C overnight. Tryptic peptides were eluted from the gel with 2 successive 20 µL volumes of 5% formic acid and concentrated in a Speedvac. (modified from Ebanks et al. (2005)). Following digestion, each sample was re-suspended in 20 µl of 0.5% formic acid. An aliquot of 3 µl of each sample was injected. A 1.5 picomole BSA gel slice stained with colloidal Coomassie blue was digested in parallel with the samples as a control for the digestion and a 3 µl injection of 20 fmol/µl BSA solution digest was used to ensure proper operation of the LC-MS-MS system. The resulting peak spectra in MGF (MASCOT generic format) were searched in MASCOT against SwissProt and most likely protein similarities reviewed. Protein score ($-10*LOG_{10}(P)$, where P is the absolute probability that the observed match is a random event, with a score of greater than 67 being significant ($p<0.05$)) along with peptide scores (probability for each matched peptide sequence) obtained through analysis were reported and compared for each band.

4.3.12 Assessment of Proteolytic Activity

Similar to preparation for protein determination, 5 µg of total protein was added to an equal volume of sample buffer solution (0.5 M Tris HCl, pH 6.8, glycerol, 10% w/v SDS. 0.2% (w/v) Bromophenol Blue). The resulting mixture was added to a 12% polyacrylamide gel containing 1% gelatin, and run at 150V for approximately 1 h at 4ºC adjacent to a broad range prestained molecular weight marker (BioRad). After separation, gels were subjected to three 10 minute washes at 4ºC with zymography wash

buffer (50 mM Tris-HCl, pH 7.5, 10% Triton X-100). Gels were removed from wash buffer and placed into incubation buffer containing 50 mM $MgCl_2$ and 6.25 mM $CaCl_2$ and incubated on a shaker at 30ºC for 19 h. After incubation, gels were stained in Coomassie blue stain for 1 h and destained (40% methanol, 10% acetic acid) repeatedly until good contrast was obtained. Gels were subsequently scanned using an 800S BIO RAD densitometer imager and resulting scans analyzed for proteolytic differences among intensity classes and over time.

4.3.13 Assessment of Enzymatic Activity

4.3.14 Lysozyme assay

Samples (25 µl) of individual oyster haemolymph, having previously been assessed for protein concentration, band differences and proteolytic activity were added to a 96 well plate in triplicate. Buffer (40 mM NaH_2PO_4, pH6.2) was prepared fresh before each assay and 50 µl added to each sample well. The resultant mixture was incubated in the plate reader for 15 min at 30ºC. Substrate (0.6 mg/ml lyophilized *Micrococcus lysodeikticus,* Sigma, in 40 mM NaH_2PO_4 buffer, pH 6.2) was also incubated separately at 30ºC for 15 min before adding 25 µl to each well containing the haemolymph and buffer mixtures and the plate was then placed immediately into the THERMOmax reader at 30ºC. The optical densities (OD) of samples were measured at 450 nm continuously (every 30 s) over one hour. Results were obtained using the software SOFTMax Pro using the initial rate of the reaction to calculate activity. One unit of activity was the amount of enzyme that catalyzed the decrease in absorbance of 0.001/min. Negative (50 µl buffer and 50 µl substrate) and positive (25 µl of chicken egg white lysozyme, 2500 units/ml Sigma) controls were run alongside samples in triplicate for each plate.

4.3.15 Alkaline Phosphatase assay

Samples (25 µl) of individual oyster haemolymph, having previously been assessed for protein concentration, band differences and proteolytic activity were added to a 96 well plate in triplicate. Buffer (100 mM ammonium bicarbonate, 1 mM $MgCl_2$, pH 7.8) was prepared fresh before each assay and 55 µl added to each sample well. The resultant mixture was incubated in the plate reader for 15 min at 30ºC. Substrate (4 mM p-nitrophenyl phosphate in 100 mM ammonium bicarbonate, 1 mM $MgCl_2$, pH 7.8) having been incubated separately at 30ºC for 15 min was added (20 µl) to each well containing the haemolymph and buffer mixtures and the plate placed immediately into the THERMOmax reader at 30ºC. The optical densities (OD) of samples were measured continuously (every 30 sec) over a one hour incubation at 405nm. Results obtained using the software SoftMax Pro using the initial rate of the reaction to calculate activity, being defined as the amount of enzyme require to release 1 pM of p-nitrophenol product in 1 min. Negative (80 µl buffer and 20 µl substrate) and positive (25 µl of alkaline phosphatase enzyme, Sigma) controls were run alongside samples in triplicate for each plate.

4.3.16 Statistical Analysis

Overall protein concentration was compared using a one way ANOVA to assess significant difference among intensity classes at each sample time as well as significant differences within intensity classes across the three sample times. Band presence in one dimensional SDS-PAGE gels were compared among intensity classes at each sample time as well as within intensity classes across the three sample times in paired analyses using the Fisher's exact test. Similarly, proteolytic band presence was compared among

groups within the initial sample period with the Heavy intensity group being compared against all others using paired Fisher's exact analyses for significance. Differences in both lysozyme and alkaline phophatase activities were compared using a one way ANOVA to assess significant difference among intensity classes at each sample time as well as significant differences within intensity classes across the three sample times.

4.4. **Results**

4.4.1 Field Infection

In 2006, naïve oysters (n = 100) deployed into the York River all became infected with *Haplosporium nelsoni,* at 100% prevalence as indicated by PCR and 61.9% prevalence by histology (Table 1). These oysters were also infected with *Perkinsus marinus* with an overall prevalence of 100% by both PCR and RFTM and 57% by histology (Table 1). Mortalities attributed to the field infection were high (79%), with subsequent collection having occurred late in the season (May deployment and August collection) and likely confounded by the mixed infections found to occur in all those oysters that survived. In 2007, naïve oysters deployed in the York River had an overall prevalence of infection with *H. nelsoni* of 100% by PCR and 88.6% by histology (Table 1). The mortalities from the field infection for 2007 were far less (21%) likely because of the timing of collection (Deployment in May and collection in July) and because oysters were not infected with *P. marinus* which occurs later in the season after the peak of *H. nelsoni* infections in this environment (Table 1).

4.4.2 Intensity Class Distribution

Infection intensities as determined by histology in both 2006 and 2007 experiments ranged from Low to High (Figure 2) and contained infections that were Local, Systemic

as well as Multi Focal in presentation. The low number of survivors from the 2006 sample disallowed for statistical strength in comparing individuals of differing intensities. Intensity classes from the 2007 sample were condensed to arrive at a comparative number of samples for protein and enzyme activity analyses (Figure 3).

4.4.3 Protein concentration

Overall average sample protein concentrations (2007) ranged from 1.48-2.21 µg/µl (Table 2). Standard deviations for the concentrations calculated among groups were rather high and indicative of overall variability among all samples. Significant differences ($p<0.05$) were found within the None intensity group which had a significantly higher average protein concentration at the pre-infection sample time (2.21 ± 0.53 µg/µl) (Table 2). Average protein concentrations also differed significantly ($p<0.05$) among intensity classes at the final harvest collection with those within the high intensity group having the highest overall average (1.79 ± 0.25 µg/µl) and the None group having the lowest (1.48 ±0.31 µg/µl) (Table 2). Both the Low and Medium intensity groups did not differ significantly over time, and among all the intensity groups no significant differences were found at the initial or two weeks post deployment samples times (Table 2).

4.4.4 Protein Profiles Comparison

4.4.5 Two-dimensional gel electrophoresis

On 2D gels of haemolymph samples from the 2007 collection contained one particular protein in high abundance that masked the presence other proteins within a given sample. This was seen on gels in the presence of a large smear (centered around pI 5-8 and MW 40-48, Figure 4) Based on the pI and MW the protein was tentatively identified as

Dominin which is the major haemolymph protein identified in oyster haemolymph (Itoh et al. 2011). The relative abundance of this protein compared with other proteins in the sample would have resulted in these other protein bands appearing at very low intensities or not at all.

The remaining constituent proteins within individual samples were not easily viewed using this method as their overall concentration within the protein samples may not have be been enough to elucidate clear protein spots using this method. Because of the lack of abundant proteins for comparison, a one dimensional gel approach was identified as more appropriate for these samples.

4.4.6 One-dimensional gel electrophoresis

Intensity groupings based on histology (2007) were run adjacent to one another for comparison on each gel and banding patterns for each group showed both repetitive proteins and varied protein profiles. No one protein was consistently observed in all representatives from a particular intensity group (Figure 5). However, when assessing the three sampling time points that were run for the representatives of each intensity class, those samples taken after field deployment (both at two weeks and two months) showed a consistent presence of protein band at ~40kD (Figure 5). This band was present in only 1 of initial haemolymph samples across all intensity classes 58% (21) of samples two weeks post deployment and 88% (32) of samples two months post deployment (Table 3). The associated band was found in 48% (13) of oysters in the none infection intensity class, in 59% (16) of oysters in low infection intensity class, 67% (18) of oysters in group three infection intensity class, and 26% (7) of oysters in high infection intensity class (Table 3). The presence of this band differed significantly ($p<0.05$) when

comparing the initial and final sample times for the None intensity group (Table 3). In both the Light and Medium intensity groups band presence differed significantly ($p<0.05$) when comparing the initial pre-infection samples with both subsequent sample times in which the band was more abundant. Within the high intensity group the final sample time displayed a significant ($p<0.05$) increase in the presence of this band when compared with the two initial samples taken from each individual of this group (Table 3). Within the second sample time, significant differences were found between the None and Medium, Low and High, and Medium and High intensity groups respectively (Table 4). Band intensities differed among proteins over the different infection intensity groupings (Figure 5), most notably the presumed Dominin protein appears less intense after field deployment in all intensity classes except the highest infection intensity class.

4.4.7 MS analysis

Protein identities for three of the four spots submitted for MS analysis yielded the protein actin as the most likely identity of the protein bands observed most consistently after field deployment (Table 5). The MW of the band (~40 kDa) is consistent with the mass of actin (42 kDa) In spots 1, 3, and 4 a strong secondary identity was also suggested in the high protein and peptide scores garnered for the dominin precursor protein (Table 5). This secondary identity was the only protein identified as originating from the host species, *Crassostrea virginica*. Protein spot 2 yielded a weak similarity with a single peptide identified to a maturase-like protein, however, this sample was likely not of the best quality and thus yielded a questionable identity (Table 5).

4.4.8 Proteolytic activity

Zymography gels assessing proteolytic activity in the haemolymph samples collected in 2007 from individual oysters over time and across different intensity classes displayed overall similar profile with the presence of a 110 kDa protease band (Figure 6). However a series of high molecular weight proteolytic bands (120 – 200 kDa) were observed only within the pre-infection initial harvest haemolymph samples and most notably overwhelmingly observed most often within those oysters that went on to develop high intensity infections (Figure 6). Comparisons of the presence of the 120-200 kDa proteolytic bands among the intensity classes yielded significant differences ($p<0.05$) when compared with the number of these bands found within the high intensity group (Table 6).

4.4.9 Enzymatic activity

Lysozyme activity was variable and did not differ significantly among the intensity groups or within the groups over time. (Table 7). Alkaline phosphatase activity did not differ significantly across intensity classes at each of the three sampling times. However significant differences of alkaline phosphatase activity were identified within all of the infected intensity classes (Low, Medium and High) which all displayed an increase in activity over time (Table 8).

4.5 Discussion and Conclusions

Protein profile and activity differences were noted in conjunction with both infection intensity as well as over time with field exposure of naïve oysters in the York River system in both 2006 and 2007. Targeting haemolymph samples from oysters over time (before, after initial exposure period and after two months of exposure) in an area in

which seasonal disease pressures are documented and closely monitored allowed these protein changes to be observed. Given the unreliable and variable results garnered from previous work in attempting to target groups of similar hosts in order to assess protein impacts, it is recommended that testing single individuals over time using non-lethal sample collection allows more readily for comparative study of MSX in eastern oyster hosts. Haemolymph is an appropriate target for testing in that it allows an individual marked oyster to be tested several times over the course of infection. When tissues are analyzed, although these are the centre of disease action as seen through histological analysis, the sacrifice of the individual is necessary. The individual variability seen in mollusks has been a challenge for proteomic research of disease, in that comparisons made between individuals from the same environment but different disease states (or infection intensities) yielded far too broad a spectrum of proteins observed among both disease state groups as well as those expressing a similar disease state (Huffman and Tripp, 1982; Auffret, 1988; McCormick-Ray and Howard, 1991; Chu and La Peyre, 1993; Ford et al., 1994; Oliver and Fisher, 1995; Bayne, 1998). In the current study, taking several samples of haemolymph from the same individual over time allowed for some control over individual variability. Because diagnostic testing requires the sacrifice of the animal in order to test tissues through PCR analysis and the observation of tissues through histological analysis, it is unclear what the disease status of the individual oysters used in this study was at the time of the second haemolymph sample (taken two weeks after initial deployment). Similarly, the overall health of the oysters from the initial sample taken after collection from a parasite-free location was not able to be discerned. Despite these unknowns, the current work suggests several interesting

changes in the haemolymph proteome associated with infection intensity and exposure over time.

The comparison of overall protein concentration displayed a significant decrease in those individuals who showed no evidence of infection with MSX at the final sample point. Many studies of *H. nelsoni* have focused on humoral factors in oysters and have reported declines in free amino acids (Feng and Canzonier, 1970) as well as total serum protein concentrations associated with systemic infections (Ford 1986). Overall serum protein concentrations have been reported to be slightly lower in *P. marinus* infected oysters compared with uninfected individuals, though not shown to be significantly different (Chu and LaPeyre,1993; LaPeyre et al., 1995), and some reports have indicted no differences seen in oysters (Chu and LaPeyre, 1989, 1993). In clams infected with *Perkinsus atlanticus,* a serum protein increase that could be due to specific polypeptides was reported by Montes et al. (1996, 1997). Decreases in overall protein were noted in the mollusk *Milanese obsolete* when parasitized with larval trematodes (Cheng et al., 1983). *Marteiliodes chungmuensis* infections have been found to reduce overall serum protein concentrations (Park, 2003; Park, 2005). Barber et al. (1988) also showed a decrease in protein content of those oysters carrying systemic MSX infections. Ford (1986) compared haemolymph protein concentration between susceptible and resistant oyster groups challenged with MSX and noted a decrease in those with the heaviest infectons. The infected oysters in this study had no significant changes in overall haemolymph protein concentrations. The decrease seen in individuals showing no histological evidence of infection could be due to energy draws and protein migration to other regions to aid in increased immune defenses or other unknown physiological needs

of these particular hosts. A similar decrease has been demonstrated in shrimp with blood proteins being diverted before moults in order to produce a new exoskeleton (Terwillister, 1999).

The comparison of haemolymph protein profiles across intensity classes over time identified a consistently observed protein band in all intensity groups occurring in the two sample points post-deployment within the MSX positive environment. Bands of this protein differed significantly with increased abundance over time in all infection intensity groups and between groups with the high intensity class showing significantly less individuals with this band. MS analysis identified actin as this band of interest which was surprising given its ubiquitous nature as an intracellular protein. Actin is a component of micro- and thin filaments utilized in the structure and motility of cells. Actin is involved in cellular functions including cell motility, shape, division, cytokinesis, muscle contraction, signaling, and the creation of cell junctions (Pratt et al., 2004). The haemolymph preparations used were cell free lysates so an increase in actin within these samples could indicate the breakdown of cellular tissue associated with disease. In an immune context, there is evidence of a role for the release of cytoplasmic actin such as suggested in neutrophil NET defenses (Neeli et al., 2009). In finfish mucus, actin has been found at significant levels and may indicate an alternate role in organisms than those traditionally identified (Easy and Ross, 2009).

The presence of high molecular weight proteases within the initial sample collection was significantly higher in those individual oysters that went on to develop high intensity infections with *H. nelsoni*. This protease activity as identified on zymograms of the host oyster haemolymph occurred only in the initial sample collection and therefore is not a

direct response to MSX infection. The finding that its abundance is greatest in those individuals who develop high intensity infections suggests that these individuals have a particular susceptibility to MSX specifically or to disease in general. This protease may indicate the presence of an underlying stressor from the collection environment which undermined the oysters' ability to deal with the subsequent assault from MSX. No consistent proteolytic changes were noted among infection intensity groups after exposure to MSX. In Perkinsosis, several studies have identified and investigated proteases involved in parasite invasion and establishment (LaPeyre et al., 1995a; Faisal et al., 1999; Garreis et al., 1996; Oliver et al., 1999; Tall 1999) as well as antiprotease host defense (MacIntyre et al., 2003; Faisal et al., 1998; Oliver et al., 1999, 2000; Romestead et al., et al., 2002; Faisal 1999; Montes et al., 1995 1996 & 1997), so the lack of evidence for proteolytic activity involved in active infection with MSX in this study is surprising. Lysozyme activity assessed over time within intensity classes as well as between classes at each sample time showed high variability and no significant trends associated with intensity or exposure to MSX over time. Lysozyme activity has been assessed in the Perkinsosis disease system with varied results either indicating increased (Chu and Le Peyre 1993a) or decreased activities (LaPeyre et al., 1995b; Garreis et al., 1996), or no association with disease (Chu and LaPeyre, 1989, 1993; Chintala et al., 1984; Chu et al., 1993). Mussel lysozyme had an inhibitory effect on *P. marinus* growth *in vitro*, far greater than serum lysozyme isolated from *C. virginica* (Anderson and Beaven, 2001). The role of environmental factors on molluscan haemolymph lysozyme levels is uncertain. The presence of high and low outliers in each intensity class impacted the

overall averages in this study accounting for much of the variability in lysozyme measures observed.

Average alkaline phophatase was similarly assess over time within and between intensity classes and increased significantly in each of the intensity classes, with the highest activities found at the final sample after two months deployment in an MSX endemic area. The group that showed no histological evidence of infection with *H. nelsoni* (but positive through PCR diagnosis) displayed only a slight increase while the other intensity classes had more pronounced increases in activity. Those in the low intensity class had a high initial activity at the time of collection with a marked decrease when sampled two weeks after deployment, followed by an increase at the final sample point. While specific roles for alkaline phosphatase activity are unknown within this system, there is evidence of a role for parasite-derived acid phosphatase in host immune response (Volety and Chu, 1997) altering bivalve cellular defense activity by disruption of phosphoproteins and inhibition of superoxide anion production. More generally, an increase in alkaline phophatase is thought to indicate stress or a trigger for an immune response (Ross et al., 2000; Iger and Abraham, 1990, 1997). The evidence of increases in relation to MSX disease infection intensity is demonstrated here suggesting a role for alkaline phosphatase in the host parasite interaction.

This work has identified several changes in the host proteome in relation to infection and *H. nelsoni* parasite intensity. The comparison of the same individual host over time, as well as targeting haemolymph as the tissue of study, allowed for reduced protein variability and therefore stronger comparative analysis. Some of the changes observed such as actin, may simply be a reflections of changes associated with other factors found

in the new environment in which the susceptible oyster were transferred. However, due to the necessity of relying on field infections it is not clear what contribution each of these factors may have had on the haemolymph proteome changes observed. Separating oysters into disease intensity classes proved an effective tool for comparison, allowing for the investigation to cover the spectrum of natural disease states. Previous work targeting disease intensity extremes (no indication of MSX infection versus heavily infected individuals) likely missed the most informative subset of disease processes, encompassing those individual hosts with energy and thus protein molecules devoted to active control and response to parasite infiltration and infection. Those individuals with the heaviest infections are likely spent with regards to immune defense molecules focusing energetics on sustaining basic biological functions. The identification of a potential proteolytic protein marker linked to a specific stressor or disease susceptibility provides an opportunity to study this interplay. Validation of this potential marker could aid in the selection of MSX-resistant strains in other locations where introduction of oysters outside of the region may be problematic and thus, could be of great use to industrial or restorative practices in identifying stocks of choice individuals.

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Figure 1: Map of Chesapeake Bay, Virginia, USA, coastline indicating collection site within the Rappahannock River (1) and deployment study site at lower York River (2).

Table 1:Prevalence of infections with MSX and Dermo as determined by histology and PCR diagnostics at the final harvest collection of oysters from the study site at lower York River, Virginia, USA from experimental trials held in 2006 and 2007. 2007 oyster collection occurred prior to infection with Dermo.

Year	Number of Oysters Deployed	Mortality % (survivors)	Prevalence of infection % MSX *Haplosporidium nelsoni*		Prevalence of Infection % Dermo *Perkinsus marinus*	
			Histology	PCR	Histology	PCR
2006	100	79% (21)	62% (13)	100% (21)	57% (12)	100% (21)
2007	100	21% (79)	88.6% (70)	100% (79)	-	-

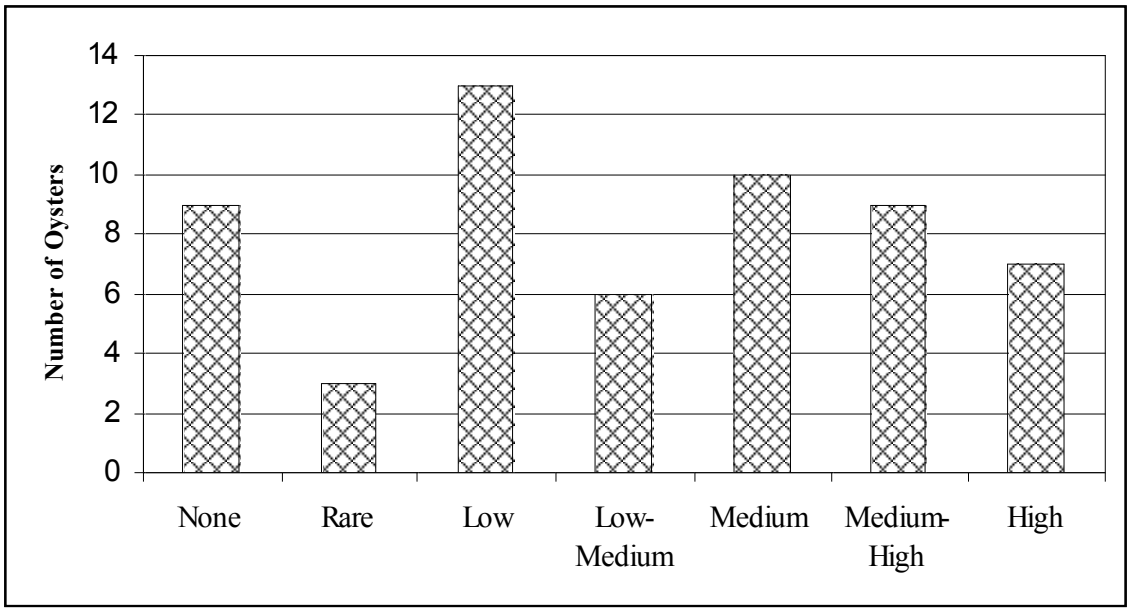


Figure 2: Distribution among intensity classes as determined through histology at final harvest of experimental oysters deployed in 2007 within the lower York River, Gloucester Point, Virginia, USA.

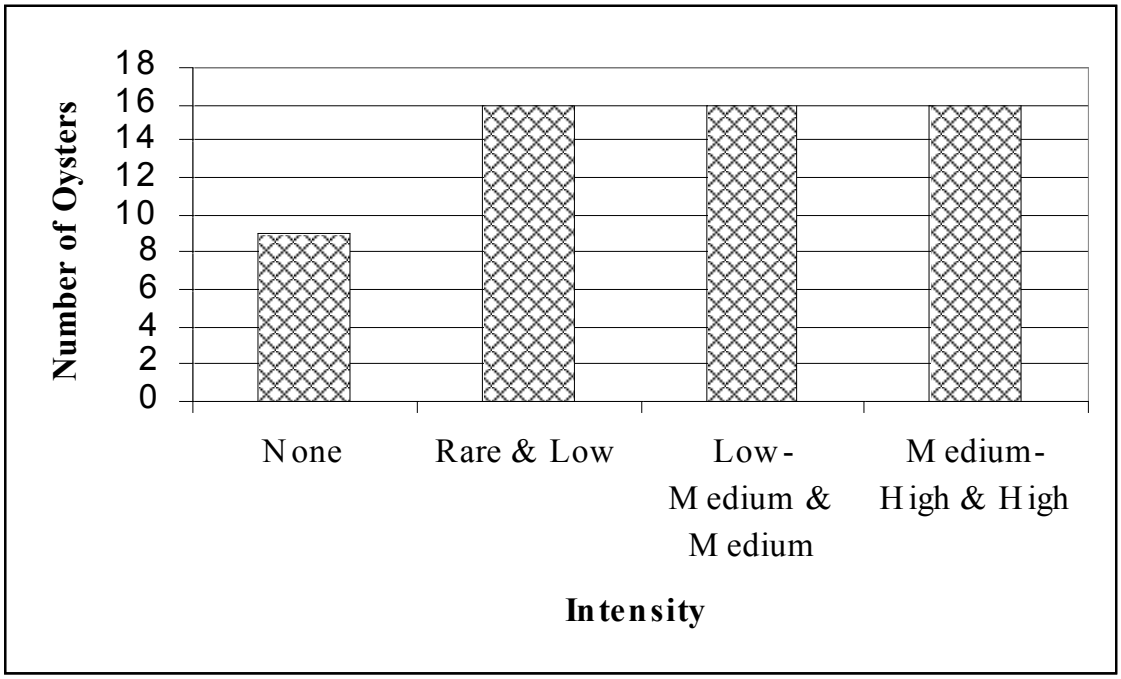


Figure 3: Intensity class groupings for analysis of protein changes, proteolysis, and enzymatic activities of oysters over course of 2007 field exposure to MSX.

Table 2: Average protein concentration (μg/μl) among the intensity classes of oysters deployed in 2007 within the lower York River, Gloucester Point, Virginia, USA at three sampling points. P-values of One way ANOVA analysis testing significant differences within intensities over time and also among intensity classes at each time point provided with * denoting significance ($P<0.05$). Average protein concentration decreased significantly over time within the group with no evidence of infection (None) group and was also significantly lower in this group compared to all other intensity groups at the final sampling point (2 months post-deployment).

Intensity	**N**	**Pre-Deployment**	**Post-Deployment (2 weeks)**	**Post-Deployment (2 months)**	**P-value**
None	9	2.21± 0.53	1.61± 0.42	1.48± 0.31	0.0033*
Rare-Low	27	1.82± 0.49	1.59± 0.34	1.68± 0.19	0.0562
Low-Medium, Medium	27	1.67± 0.45	1.60± 0.31	1.62± 0.26	0.7165
Medium-High, High	17	1.80± 0.59	1.81± 0.20	1.79± 0.25	0.9834
P-value	-	0.0604	0.1007	0.0231*	-

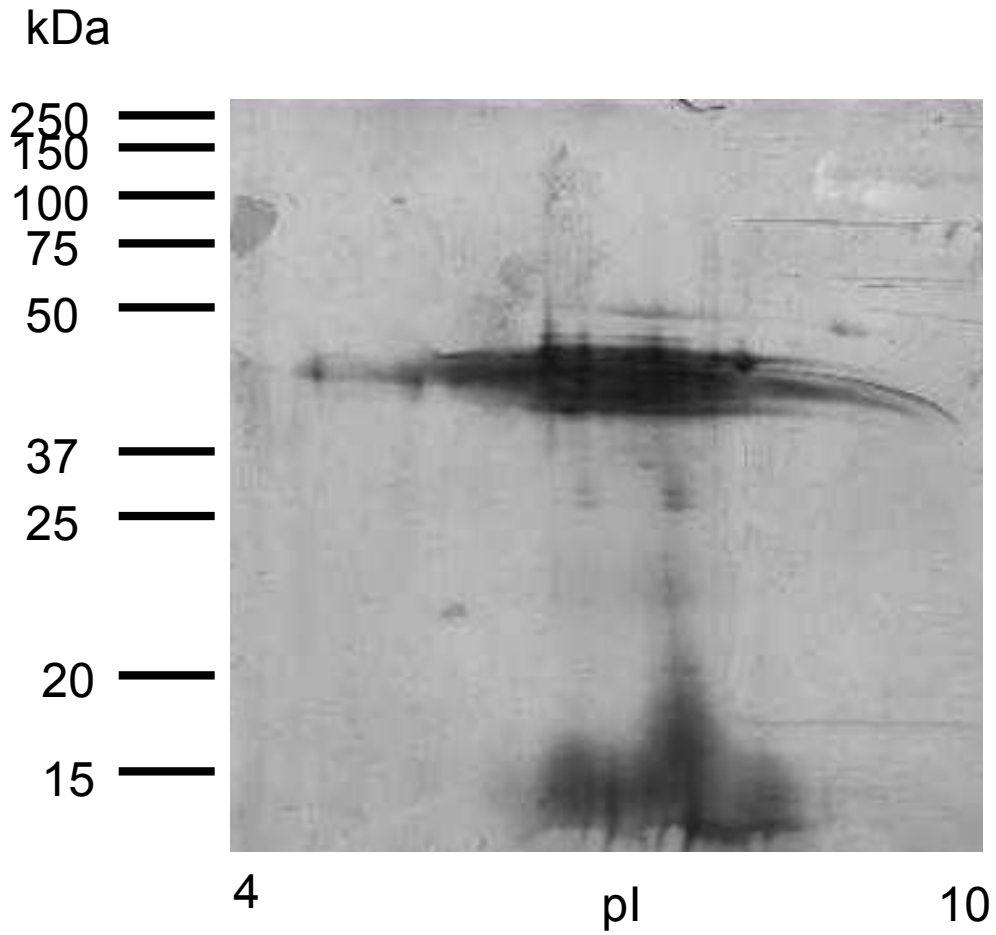


Figure 4: Protein profile of haemolymph protein (200 ug) collected from one individual oyster run on a two dimensional electrophoresis gel (14%) and silver stained for analysis.

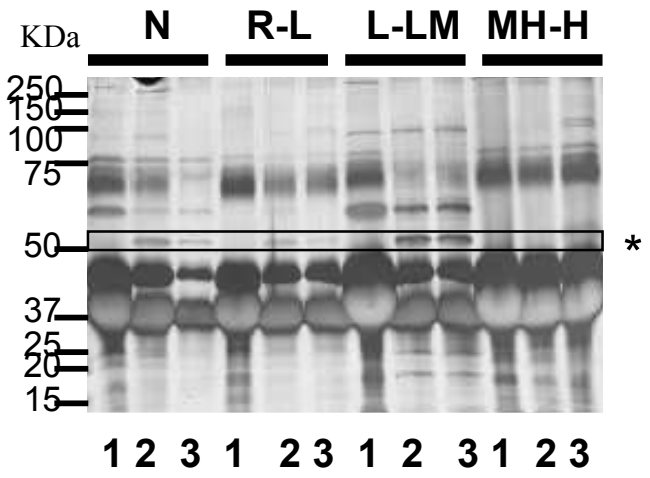


Figure5: SDS Page gel of haemolymph from four individual oysters deployed within the lower York River, Gloucester Point, Virginia, USA. One individual representing each intensity class (N: None, R-L: Rare and Low, LM-M: Low-Medium and Medium, MH-H: Medium-High and High) at each of the sampling points (1-Initial collection, 2- two weeks post deployment in experimental field conditions, 3 – final harvest from field).

Table 3: Abundance of protein band of interest among intensity classes and across sampling times with the comparison of abundance over time within intensity classes using the Fisher exact test, * denotes significance ($p<0.05$)

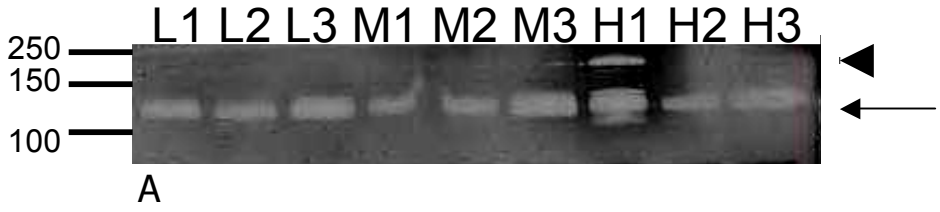
Intensity Class	Number samples with protein band (~40kD) / total samples			Comparison with over time Fisher's Exact p-value		
	T1:Pre-deployment	T2:Two weeks post-deployment	T3:Two months post-deployment	T1 - T2	T1 - T3	T2 - T3
None	1/9	4/9	8/9	0.2941	0.0034*	0.1312
Rare-Low	0/9	7/9	9/9	0.0023*	<0.0001*	0.4706
Low-Medium &Medium	0/9	9/9	9/9	<0.0001*	<0.0001*	-
Medium-High & High	0/9	1/9	6/9	1.000	0.0090*	0.0498*

Table 4: Comparison of abundance of protein band of interest among intensity classes at the final sample point, two months post deployment using a Fisher's exact test, * denotes significance (<0.05).

Intensities compared	Comparison between intensities at T3 Fisher's Exact p-value
None - Low	0.3348
None - Medium	0.0294*
None - High	0.2941
Low - Medium	0.4706
Low - High	0.0152*
Medium - High	0.0004*

Table 5: Identities yielded from Mass Spectrometry analysis and subsequent Mascot search of SwissProt database. Each excised band reports a most likely identity based upon overall protein score ($-10*LOG_{10}(P)$, where P is the absolute probability that the observed match is a random event, with a score of greater than 67 being significant ($p<0.05$)), number of similar peptides and individual peptide scores, predicted mass and original species from which protein was described. Secondary protein matches are also provided.

Spot	Intensity class	Sample Time	ID	Protein Score	Peptides	Species	Mass
1	None	T3 -final harvest	1-cytoskeletal actin IIIa	114	3 -34 -57 -29	*Strongylocentrotus purpuratus*	42024
			2- dominin precursor	59	2 -58 -59	*Crassostrea virginica*	21270
2	Rare-Light	T3 -final harvest	1-maturase-like protein	61	1 -61	*Adesmia volckmannii*	61546
3	Light-Medium & Medium	T2 – Two weeks post deployment	1-actin	127	3 -46 -47 -34	*Pterosperma cristatum*	38554
			2-actin	123	3 -46 -47 -39	*Oxystele tigrina*	24943
			3-dominin precursor	114	3 -27 -76 -38	*Crassostrea virginica*	21270
4	Medium-High & High	T3 -final harvest	1-cytoplasmic actin	343	8 -43 -57 -42 -28 -22 -37 -64 -55	*Dreissena polymorpha*	42168
			2- dominin precursor	85	3 -40 -55 -31	*Crassostrea virginica*	21270



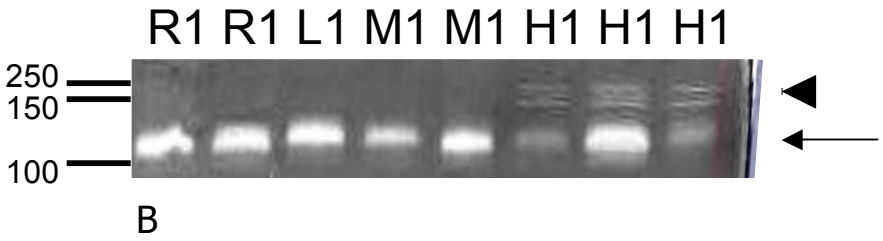


Figure 6: Zymography gel (A) of haemolymph samples from three individual oysters representing three intensity classese (L- Rare-Low, M- Low Medium- Medium, and H – Medium-High and High) sampled at three time points. Zymography gel (B) of haemolymph samples from eight individual oysters representing four intensity classes (R-Rare, L-Low, M-Medium and H- High) all taken from the initial sampling time, pre-deployment exposure to MSX infected water system. Arrow indicates ubiquitous 110 kDa protease band, arrowheads identify high molecular weight bands (140-200 kDa).

Table 6: Number of individual oysters with high MW (140-200 kDa) proteolytic bands in each intensity class, from the first sample collection pre-infection in 2007. Tests for significance between numbers of bands found in highest intensity class compared with all other intensity classes are provided, * denotes significance $p<0.05$.

Intensity Class	Number of pre-deployment samples with proteolytic bands (~150kD) / total samples	Comparison with Medium-High & High Intensity Class Chi Square, df, p-value	Comparison with Medium-High & High Intensity Class Fisher's Exact p-value
None	0/9	9.375, 1, p=0.0022*	p= 0.0028*
Rare-Low	1/16	11.22, 1, p=0.0008*	p= 0.0021*
Low-Medium & Medium	1/16	11.22, 1, p=0.0008*	p= 0.0021*
Medium-High & High	10/16	-	-

Table 7: Average Lysozyme activity (units of activity/ug of protein) from haemolymph taken from oysters belonging to each intensity class at each of the three sampling points (1-pre-infection, 2- two weeks post deployment in experimental field conditions, 3 – final harvest from field). P-values of One way ANOVA analysis testing significant differences within intensities over time and also among intensity classes at each time point provided with * denoting significance, $p<0.05$.

Intensity	**Pre-Deployment**	**Post-Deployment (2 weeks)**	**Post-Deployment (2 months)**	**P-value (over time)**
None	772.2±475.9	702.4±556.5	593.4±435.7	0.7422
Rare (3)	438.5±307.8	583.2±478.9	635.5±582.5	0.8711
Low	905.4±858.3	1374.5±1893.2	705.4±555.3	0.3800
Low/Medium	1483.3±1787.6	795.2±800.1	971.1±927.3	0.6221
Medium	1140.8±727.3	629.0±529.2	742.3±585.9	0.1452
Medium/High	817.0±792.6	836.8±858.8	661.7±510.2	0.8592
High	799.4±651.4	466.3±249.0	502.8±449.2	0.3810
P-Value (among intensities)	0.6209	0.5794	0.8393	-

Table 8: Average Alkaline Phosphatase activity (units of activity/ug of protein) from haemolymph taken from oysters belonging to each intensity class at each of the three sampling points (1-pre-infection, 2- two weeks post deployment in experimental field conditions, 3 – final harvest from field). P-values of One way ANOVA analysis testing significant differences within intensities over time and also among intensity classes at each time point provided with * denoting significance, $p<0.05$.

Intensity	**Pre-Deployment**	**Post-Deployment (2 weeks)**	**Post-Deployment (2 months)**	**P-value**
None	0.0002±0.03	0.001±0.07	0.103±0.07	0.0557
Rare - Low	0.407±0.03	0.002±0.04	0.767±0.66	0.0003*
Low/Medium - Medium	0.016±0.04	0.334±0.97	3.10±3.7	0.0125*
Medium/High – High	0.073±0.14	0.020±0.04	1.94±1.7	0.0004*
P-Value	0.3935	0.4998	0.0916	-

Chapter 5:

General Discussion

Review of Research Goals and Findings

The study of host parasite interactions of parasitic protozoans involves the use of specialized techniques in order to obtain information about each system. In depth study can be achieved through manipulation of parasitic species in the laboratory through the use of culture or the establishment of models fulfilling lifecycle and environmental requirements allowing for experimental infection of hosts to be studied. In the case of *Haplosporidium nelsoni,* study relies on field investigation or the culture of wild infected oysters because the parasite's life cycle remains unknown and the organism cannot be cultured *in vitro* nor transmitted *in vivo* experimentally. Given these limitations, the diagnosis of *H. nelsoni* infections is of particular importance in order to properly identify the parasite and assess appropriate hosts for use in the study of the disease relationship. The biochemical interactions between parasites and their hosts can be incredibly informative as they are filled with specific parasite factors relating to initial infiltration, infection, disease progression, as well as defense strategies employed by both the host and parasite during this interplay. Biochemical interactions can be investigated using several methods, from holistic, proteomics approaches that examine changes in all of the constituent proteins found in a tissue or sample type in response to a change or investigated using a more narrowly defined set of parameters, such as specific enzyme activities. Total protein profiles of specific tissues collected from the host can be assessed through techniques such as SDS-PAGE, which separates all the proteins in a given sample based on molecular weight or 2-DGE which utilizes this separation technique along with the added benefit of separation of proteins based on their isoelectric point. As well, electrophoretic methods may be useful in examining enzyme profiles.

More specifically, proteolytic activity of individual samples can be assessed through the using zymography and additionally specific enzyme activities can be measured using substrate assays. The combination of the protein approaches outlined, when targeted towards a given tissue, provide a comprehensive investigation of the proteome changes within the host parasite interaction.

With the spread of *H. nelsoni* to Canadian waters in the Bras d'Or Lakes, the current investigation first assessed the spread and detection of this parasite within host oyster, *Crassostrea virginica*, populations in three localities in this new landscape of disease, the Bras d'Or Lakes. Secondarily, this study set out to investigate protein interactions in this host-parasite system in order to identify potential biomarker targets involved in populations with and without disease to gain further information about this pathogen and to aid in understanding of the disease process that follows infection. The host populations sampled were also used to assess the use of infected and uninfected tissues as targets for protein and enzymatic analysis with the goal of identifying proteins specific to the disease state. As part of the study, field experiments were carried out within the established disease environment of Chesapeake Bay, Virginia, in order to describe protein profiles of host haemolymph comparing naïve and infected samples in order to identify any changes correlating with disease.

Contributions and limitations

The populations studied in the Bras d'Or Lakes provide a snapshot of MSX disease spread within a new environmental range. The continued evidence of spread and infection within the Lakes further illustrates that study of this parasite within this

particular population is needed. The initial goal of the work within the Lakes was to identify the population at risk and to illustrate how widespread the pathogen may have become in just a few short years since its initial detection. Sampling of large numbers at sites in which the parasite had not yet been detected or had limited diagnostic positives in previous years had been expected to show low prevalence at these locations displaying the spread of the pathogen had likely occurred throughout the lakes system. Test results however provided an unexpected outlook of MSX within the Lakes. Nyanza Bay had continued infection at historic levels ever since introduction of the parasite. East Bay, which had been targeted as an area likely to have low prevalence levels, instead had a prevalence similar to that seen in Nyanza, despite the appearance of a healthy multi-year class population at the time of collection. Most interesting was the collection made at Lynches River, which showed no indication of infection when assessed through histology, but had a high prevalence (28%) when PCR was carried out. This large discrepancy among the two diagnostics tests used to identify and quantify the presence of MSX within these populations provided evidence that there may be factors contributing to the lack of disease establishment within this population despite infection. These factors may lie within the host population at this location or within the environment in which they live or some combination of both. Further study of this and nearby populations in particular would be of great value in the investigation of *H. nelsoni* within the Bras d'Or Lakes, along with the study of physical environmental parameters at various locations in the Lakes and may help determine a correlation between environmental factors and the likelihood that an infection will progress to a disease state.

Comparison of digestive gland, gill and mantle tissues from infected and uninfected oyster using protein electrophoresis proved too variable for use in identification of protein expression differences associated with disease. However, the methodologies employed in this attempted study provide an excellent set of tools to pursue a specific protein target within these tissues. Separation utilizing two-dimensional electrophoresis was greatly improved through initial dialysis of samples. A pre-fractionation method developed for use in cultured cells to identify proteins located in cytosolic, membrane and nuclear components was employed and allowed for the elucidation of numerous high molecular weight proteins that were otherwise missed through use of traditional one dimensional SDS-PAGE and two-dimensional gel electrophoresis methods. An increase in sample size through initial screening using SDS-PAGE may help in controlling for the variability seen among individual oysters. Targeting a single tissue may also further reduce variability, rather than using a cross section of the three tissues used for MSX disease diagnosis, using the new methods with which to study the tissue proteins of oyster hosts presented in this work.

The high degree of variability encountered through the targeting of host tissues from different individuals based on disease state identified the need to reduce variability. One means to do this is to compare tissues from the same individual over the course of infection. Within the Chesapeake Bay, populations of infected oysters are closely monitored and the infection pressure is tracked allowing for an accurate estimation of the seasonality of infection and disease development in this system. A collection of naïve oysters was achieved due to the existence of environmental constraints of certain populations that are free from infection with MSX. Haemolymph as a target tissue for

protein analysis allowed naïve individual oysters to be bled repeatedly with an initial collection prior to infection being compared to subsequent haemolymph samples from the same individual after their deployment in the MSX pressured environment. Histology with PCR then allowed for determination of both infected and uninfected individuals as well as a snapshot of disease intensity at the final sample point This allowed for groupings of individual oysters at all sample times based on the final disease intensity level. Overall variability was reduced in the comparison of single individuals over three representative time points. The use of haemolymph as the target tissue also yielded a lower overall abundance of protein in each sample, restricting the 2-DGE analysis and therefore the use of one dimensional SDS-PAGE for protein comparison was used resulting in a highly effective approach to the study of MSX targeting host haemolymph proteins.

Using the above approach, several specific protein correlations were observed within this host parasite system. The first was overall protein concentration found within individual haemolymph samples collected over the course of the field investigation. Overall protein concentration decreased significantly within the group with no evidence of infection through histology but positive by PCR analysis at the time of the final sample. The reduction in overall protein concentration was specific to this group and was also not found to differ significantly across intensity groupings. This may suggest a draw on defensive proteins needed to ward off potential infections resulting in a successful immune response or a healthy oyster in which proteins are diverted to gonad development. Significant changes in overall protein concentration of challenged oysters

that mitigate MSX infection and disease could be pursued through targeted study of these individuals at the end of the field infection cycle.

The only consistent change in protein bands identified by SDS-PAGE which correlated to exposure to an MSX positive environment was present in nearly all individuals regardless of intensity at the final sample time. This protein band was absent initially in naïve oysters but present in both the two-week post-deployment and two-months post deployment haemolymph collections. Identification through MS analysis yielded actin as the most likely protein identity for this band. The increase in actin within the cell free lysates of the haemolymph collections made post-deployment could be a result of a degradation of tissues involved in disease or a directed release of actin on its own or along with other cytoplasmic proteins as a targeted defensive strategy. As such this finding may indicate the identification of a role for actin in the disease manifestation of MSX in the eastern oyster. There may also exist a purely environmental influence resulting in the increase of actin, unrelated to MSX specifically, and perhaps involved in environmental changes encountered after deployment in this new location such as change in salinity.

In assessing the proteolytic activity within the haemolymph from different infection intensity class groups, a series of high molecular weight proteolytic bands were identified in the naïve oyster initial collection prior to exposure to MSX. This band correlated significantly with the development of high intensity infections. It is the first evidence of a marker indicating susceptibility of *C. virginica* to the development of high intensity disease with the MSX parasite. It is unknown if this factor is an indicator of genetic susceptibility, environmental susceptibility, or that this group of oysters had a specific

pre-existing condition weakening their defenses against any immune threat. However, further study is warranted to establish the action of this protease as well as its role in this disease system. Insights into the role of this proteolytic activity linking certain susceptible oysters at risk of developing high intensity infections could lead to important information regarding the disease process and cycling of this parasite.

Through investigation of certain enzymatic activities, alkaline phosphatase was identified as significantly more active over time within intensity class groupings, as well as displaying significantly higher activity among medium and heavy intensities. The particular role for this enzyme is not known but provides an interesting study target, as well as an excellent candidate for stress screening in oyster stocks using haemolymph as a non-lethal target tissue. Evidence of increased alkaline phosphatase activity correlated to infection intensity and disease progression over time is novel in the study of this host parasite system.

The multi-faceted approach to understanding the proteins involved in the MSX disease interaction within the eastern oyster led to the development of a method with which to study the parasite within the field, which minimizes individual variability and allows for comparison across intensities found within an infected population. The specific protein changes and findings can be used as a starting point toward further characterization of these changes and their relationship to the specific actions of disease and/or defense within this host parasite system.

Research futures based on present findings

Assessing an individual over time greatly increased the ability to identify protein differences in this system. If SDS-PAGE or 2-DGE were to be tested in the future,

targeted isolation and screening of specific samples should involve separation of individual tissues. Using the approach of naïve individuals deployed in environments of disease pressure and assessing haemolymph over time within the Bras d'Or Lakes would allow for more information about what is impacting the development of disease in Lynches River. For example individuals from Lynches River could be deployed in Nyanza Bay at specific times of the year. There should also be an assessment of those oyster populations who have dealt with disease pressure for many years (individuals from Nyanza) over time in order to look for changes to identify immune molecules that could be involved in successful mitigation of disease. The specific targets identified provide many avenues for continued investigations in the Virginian and the Bras d'Or Lakes environments and valuable comparison of differences between these two areas with one being well established in dealing with this parasite for over 50 years and the other having new pressures and continued spread. Taking some of the information established by the current work and incorporating a genetic approach to investigate expression of specific genes would further describe the biochemical interactions of this host parasite system. *Haplosporidium nelsoni* presents many challenges in the study of its success within certain environments, key changes in the proteome of host haemolymph provide some direction in continuing to discover the elements involved in infection and disease in this relationship.